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(54) Title: CAROTENOID BIOSYNTHESIS

(57) Abstract: The invention provides materials and methods that can be used to make carotenoids having greater than 40 carbon atoms (C>40). The invention also provides isolated nucleic acid molecules that encode polypeptides that allow C40 carotenoids to be converted to C50 carotenoids. The isolated nucleic acid molecules can be introduced into production cells, wherein the production cell becomes capable of the biosynthesis and the conversion of the C>40 carotenoids.

CAROTENOID BIOSYNTHESIS

FIELD OF THE INVENTION

This invention relates to materials and methods for making carotenoids.

BACKGROUND

Carotenoids have significant utility in pigment and anti-oxidant applications. For example, many of the red, yellow, and orange colors observed in nature are pigments provided by one or more carotenoids. Carotenoids are among the best antioxidants provided by nature—orders of magnitude better than other naturally available materials such as vitamin C or vitamin E. The carotenoid molecule comprises multiples of the isoprene molecule, a C5 hydrocarbon with two double bonds. In view of the dual unsaturation of the isoprene molecule, the class of carotenoid molecules is characterized by long organic chains with conjugated double bonds. It has been shown that the high antioxidant capacity and the vivid pigmentation are directly attributable to the long chains of conjugated double bonds. For example, Conn *et al. J. Photochemistry Photobiology B*, **11**: 41-47, 1991 compared the common β-carotene—a C40 carotenoid having 11 conjugated double bonds -- with a chemically synthesized C50 β-carotene having 15 conjugated double bonds and with a chemically synthesized C60 β-carotene having 19 conjugated double bonds. The Conn *et al.* study concluded, based on quenching of singlet oxygen, that the efficiency of antioxidant activity increased with increasing numbers of conjugated double bonds.

The literature is replete with details concerning the biosynthesis of C40 carotenoids, including details concerning the associated genes and the enzymes encoded by the genes. However, the biosynthesis and biochemical properties of C>40 carotenoids is poorly understood relative to the level of knowledge of C40 carotenoids. Ironically, C>40 carotenoids have the potential to be more effective antioxidants, to provide greater health benefits, and to generate novel improved colored pigments (i.e. pigments of longer wavelength absorbance maxima).

There are numerous reports in the literature of bacteria that are capable of producing C50 carotenoids. Examples of such bacteria include *Halobacterium salinarium, Cellulomonas biazotea, Arthrobacter glacialis, Corynebacterium poinsettiae,*

Micrococcus luteus, and *Agromyces mediolanus.* Examples of C50 carotenoids produced by *Micrococcus luteus*, *Agromyces mediolanus*, and *Halobacterium salinarium* are shown in FIG 11.

Three C50 carotenoids (molecular formulae $C_{50}H_{72}O_2$) have been isolated from the psychrophilic bacterium *Arthrobacter glacialis*, including bicyclic decaprenoxanthin, aliphatic bisanhydrobacterioruberin, and monocyclic A.g. 470 (Arpin N, *et al. Acta Chem Scand B* **29**:921-6, 1975).

It is clear that carotenoid characteristics such as antioxidant and pigment capabilities improve with a greater number of conjugated double bonds. In view of production and other technical limitations, however, commercial use of carotenoids has been substantially limited to those no longer than C40. To allow sufficient production of the C50 carotenoid to commercially utilize its improved properties, it would be desirable to have the capability to convert C40 carotenoids to C50 carotenoids by genetic manipulation.

SUMMARY OF THE INVENTION

The present invention is based on isolated nucleic acid molecules that encode polypeptides that allow C40 carotenoids to be converted to carotenoids having greater than 40 carbon atoms (C>40), such as a C50 carotenoid. These polypeptides can be used *in vitro* or *in vivo*. The isolated nucleic acid molecules can be introduced into a production cell, wherein the production cell becomes capable of converting a C40 carotenoid to a C>40 carotenoid, such as a C50 carotenoid.

In one aspect, the invention features an isolated polypeptide, isolated nucleic acid molecules encoding the polypeptide, and production cells that include the isolated nucleic acid molecules. The isolated polypeptide includes at least one amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; (b) an amino acid sequence having at least 10 contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; (c) an amino acid sequence having one or more conservative amino acid substitutions within the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26;

and (d) an amino acid sequence having at least 65% sequence identity with the amino acid sequences of (a) or (b). Polypeptides at least 10 amino acid residues in length are useful for, among other things, generating specific binding agents, such as antibodies. Polypeptides having at least 65% sequence identity with the amino acid sequences of (a) or (b) are useful for creating specific binding agents that vary in binding strength, as well as for creating polypeptides with enzymatic activities that vary in binding strength (Km) and/or turnover rate (Kcat).

The nucleic acid molecule can encode a polypeptide capable of converting a C40 carotenoid to a C50 carotenoid, a C40 carotenoid to a C45 carotenoid, a C45 carotenoid to a C50 carotenoid, or capable of synthesizing a C40 carotenoid. These polypeptides can be used *in vitro* or *in vivo*.

The invention also features an isolated nucleic acid molecule or a production cell containing the nucleic acid molecule. The nucleic acid molecule includes a nucleic acid sequence selected from the group consisting of: (a) the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23; (b) a nucleic acid sequence having at least 10 contiguous nucleotides of the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23; (c) a nucleic acid sequence that hybridizes under moderately stringent conditions to the nucleotide sequence of (a); and (d) a nucleic acid sequence having 65% sequence identity with the nucleic acid sequence of (a) or (b). These nucleic acid molecules are useful for identifying other nucleic acid sequences that encode polypeptides with similar enzymatic activities to those described herein. Methods such as the polymerase chain reaction (PCR), which utilizes short fragments of the disclosed sequences, or Northern and/or Southern blotting procedures which utilize slightly longer fragments, can be used to identify substantially similar sequences.

In another aspect, the invention features a method for making a C50 carotenoid. The method includes contacting at least one of the polypeptides described above with a C40 carotenoid such that the C50 carotenoid is made. A C50 carotenoid also can be made by culturing the production cell described above under conditions wherein the C50 carotenoid is made.

In yet another aspect, the invention features a method for making a C45 carotenoid. The method includes contacting at least one of the polypeptides described above with a C40 carotenoid such that the C45 carotenoid is made. A C45 carotenoid also can be made by culturing the production cell described above under conditions wherein the C45 carotenoid is made.

The invention also features a method for making a polypeptide. The method includes culturing the production cell described above under conditions such that the polypeptide is made.

In another aspect, the invention features a specific binding agent that binds to the polypeptide described above.

In yet another aspect, the invention features a method for making a C>40 carotenoid. The method includes culturing a production cell, wherein the production cell includes an exogenous nucleic acid molecule, wherein the exogenous nucleic acid molecule encodes a polypeptide that elongates a C>40 carotenoid by at least one carbon atom, wherein the product produced by the polypeptide is a carotenoid having a carbon backbone of >40 carbon atoms. The use of the term carbon backbone refers to the single contiguous chain of carbon-carbon bonds that are found in carotenoids. The exogenous nucleic acid molecule can include a nucleic acid sequence selected from the group consisting of: (a) the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23; (b) a nucleotide sequence having at least 10 consecutive nucleotides of the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23; (c) a nucleic acid sequence that hybridizes under moderately stringent conditions to the nucleotide sequence of (a); and (d) a nucleic acid sequence having 65% sequence identity with the nucleic acid sequence of (a) or (b). The exogenous nucleic acid molecule can encode a polypeptide, wherein the polypeptide includes an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; (b) an amino acid sequence having at least 10 contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; (c) an amino acid sequence having one or more conservative amino acid substitutions within the amino acid sequence of SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20,

24, 25 or 26; and (d) an amino acid sequence having at least 65% sequence identity with the amino acid sequences of (a) or (b).

These and other aspects of the invention will are discussed in more detail in the following detailed description.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter codes for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

SEQ ID NO: 01 is the nucleic acid sequence for the *A. mediolanus lctA* gene (a lycopene cyclase).

SEQ ID NO: 02 is the nucleic acid sequence for the *A. mediolanus lctB* gene.

SEQ ID NO: 03 is the nucleic acid sequence for the *A. mediolanus lctC* gene.

SEQ ID NO: 04 is the amino acid sequence encoded by SEQ ID NO: 01.

SEQ ID NO: 05 is the amino acid sequence encoded by SEQ ID NO: 02.

SEQ ID NO: 06 is the amino acid sequence encoded by SEQ ID NO: 03.

SEQ ID NO: 07 is the nucleic acid sequence for the *M. luteus lctA* gene.

SEQ ID NO: 08 is the nucleic acid sequence for the *M. luteus lctB* gene.

SEQ ID NO: 09 is the nucleic acid sequence for the *M. luteus lctC* gene.

SEQ ID NO: 10 is the amino acid sequence encoded by SEQ ID NO: 07.

SEQ ID NO: 11 is the amino acid sequence encoded by SEQ ID NO: 08.

SEQ ID NO: 12 is the amino acid sequence encoded by SEQ ID NO: 09.

SEQ ID NO: 13 is the nucleic acid sequence for the *A. mediolanus idi* gene.

SEQ ID NO: 14 is the nucleic acid sequence for the *A. mediolanus crtE* gene.

SEQ ID NO: 15 is the nucleic acid sequence for the *A. mediolanus crtB* gene.

SEQ ID NO: 16 is the nucleic acid sequence for the *A. mediolanus crtI* gene.

SEQ ID NO: 17 is the amino acid sequence encoded by SEQ ID NO: 13.

SEQ ID NO: 18 is the amino acid sequence encoded by SEQ ID NO: 14.

SEQ ID NO: 19 is the amino acid sequence encoded by SEQ ID NO: 15.

SEQ ID NO: 20 is the amino acid sequence encoded by SEQ ID NO: 16.

SEQ ID NO: 21 is the nucleic acid sequence for the *M. luteus crtE* gene.

SEQ ID NO: 22 is the nucleic acid sequence for the *M. luteus crtB* gene.

SEQ ID NO: 23 is the nucleic acid sequence for the *M. luteus crtI* gene.

SEQ ID NO: 24 is the amino acid sequence encoded by SEQ ID NO: 21.

SEQ ID NO: 25 is the amino acid sequence encoded by SEQ ID NO: 22.

SEQ ID NO: 26 is the amino acid sequence encoded by SEQ ID NO: 23.

SEQ ID NOS: 27-30 are primers used to amplify regions of the carotenogenic operon from the Y1 clone.

SEQ ID NOS: 31 and 32 are primers used to amplify ORFY.

SEQ ID NO: 33 is a primer used in combination with SEQ ID NO: 32, to amplify the region of *A. mediolanus* genomic DNA containing the X1, X2, and Y ORFs.

SEQ ID NOS: 34 and 35 are primers used to amplify a mutated ORFX1, ORFX2, and ORFY fragment.

SEQ ID NOS: 36 and 37 are primers used to amplify a mutated ORFX2 fragment.

SEQ ID NOS: 38 and 39 are primers used to amplify a mutated ORFY fragment.

SEQ ID NOS: 40 and 41 are primers used to make a probe to identify *M. luteus* homologs.

SEQ ID NOS: 42-45 are primers used for *M. luteus* genomic walking.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 is the nucleotide sequence of the 9-Kb Y1 operon – the C50 carotenoid producing operon from *A. mediolanus*.

FIG 2 contains HPLC chromatograms of carotenoid extracts from *A. mediolanus*, *E. coli* transformed with the *idi*-Y construct, *E. coli* transformed with the *idi-crtI* construct, a lycopene standard, and *E. coli* transformed with the *idi*-X2 construct.

FIG 3A contains chromatograms of carotenoid extracts from *A. mediolanus* and *E. coli* transformed with the *idi*-ORFY construct (Yellow *E. coli* clone Y33). The two analyses show a peak at virtually the same retention time.

FIG 3B contains visible spectra for the *A. mediolanus* extract and an extract from *E. coli* transformed with the *idi*-ORFY (Yellow *E. coli* clone Y33). The visible spectra for both peaks are virtually identical.

FIG 4 is mass spectra of carotenoid extracts from *A. mediolanus* and from *E. coli* transformed with the *idi*-ORFY construct (Yellow *E. coli* clone Y33). The analysis confirmed that the compound from clone Y33 and *A. mediolanus* at a retention time of 7 minutes had the same mass.

FIG 5 contains HPLC chromatograms of carotenoids extracted from *E. coli* transformed with the *idi-crtI* construct and a lycopene standard (Sigma).

FIG 6 contains visible spectra for carotenoids extracted from *E. coli* transformed with the *idi-crtI* construct and a lycopene standard (Sigma). The visible spectra are virtually identical.

FIG 7 contains mass spectra of a lycopene standard, carotenoids produced in *E. coli* transformed with the *idi-crtI* construct and carotenoids produced in *E. coli* transformed with the *idi*-ORFX2 construct.

FIG 8 is a visible-spectrophotometric analysis of carotenoid extracts from *A. mediolanus* and mutant *E. coli* clones. The mutant *E. coli* clones produced the C40 carotenoid lycopene and no C50 carotenoid, while *A. mediolanus* produced the C50 carotenoid decaprenoxanthin.

FIG 9 is a schematic of the arrangement of genes within the biosynthetic pathway for the production of a C50 carotenoid for *A. mediolanus*, *M. luteus*, *C. glutamicum*, *H. salinarium*, and *M. thermoautotrophicum*.

FIG 10 is a schematic of the biosynthetic pathway for the production of decaprenoxanthin in *A. mediolanus* and the postulated role of the *lctA*, *lctB*, and *lctC* genes.

FIG 11 depicts examples of C50 carotenoid structures reported in the literature.

FIG 12 is the nucleotide sequence of the C50-carotenoid producing operon from *M. luteus* ATCC 383.

DETAILED DESCRIPTION

I. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, Genes VII, Oxford University Press, 1999 (ISBN 0-19-879276-X); Kendrew et. al. (editors), The Encyclopedia of Molecular Biology, Blackwell Science Ltd., 1994 (ISBN 0-632-021182-9); and Robert A. Meyers (editor), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, BCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

Carotenoid – A molecule that includes at least two isoprenoid units joined in such a manner that the two joined isoprenoid units have two methyl groups in a 1,6-positional relationship. The term "carotenoid" also includes derivatives having one or more hydrogen atoms replaced with a substituent group or atom. Non-limiting examples of substituents include 1) hydroxyl groups (yielding an alcohol); 2) methoxyl groups (derived from an alcohol); 3) glycosyl (sugar) residues (attached by an ether bond); 4) fatty acid residues (attached by an ester bond); 5) carbonyl groups (yielding aldehydes or ketones); 6) sulfates; 7) carboxylic acids; and 8) epoxides. Additional carbon atoms can be added via the substituent group. Hydrogen atoms can be replaced anywhere on the molecule, including within the methyl groups in the 1-6 positional relationship. Non-limiting examples of typical carotenoids include β-carotene, phytoene, lycopene, dehydrogenans P-452, decaprenoxanthin, 4,4'-diapophytoene, and norbixin.

CX – The carotenoid molecules of the present application are characterized by the term "CX", wherein "C" refers to carbon atoms and the "X" refers to the total number of carbon atoms in the isoprenoid units of the carotenoid molecule.

C>X – The designation "C>X carotenoid" means a carotenoid having more than X carbon atoms total in the isoprenoid units of the carotenoid molecule. Similarly C<X is used to identify a carotenoid having less than X carbon atoms.

Homology – A term referring to the sequence identity between two or more sequences.

Isoprenoid – A molecule that is a multiple of the C5 hydrocarbon isoprene (2-methyl-1,2-butadiene).

Polypeptide - The term "polypeptide" includes any chain of amino acids at least eight amino acids in length, regardless of post-translational modification.

Nucleic acid - The term "nucleic acid" as used herein encompasses both RNA and DNA including, without limitation, cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

Isolated – The term "isolated" as used herein with reference to a polypeptide refers to a polypeptide that has been separated from the cellular components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, 92%, 95%, 98%, or 99%), by weight, free from proteins and naturally-occurring organic molecules that are naturally associated with it. In general, an isolated polypeptide will yield a single major band on a non-reducing polyacrylamide gel.

The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-

occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

It will be apparent to those of skill in the art that a nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated nucleic acid.

Exogenous: The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, non-naturally-occurring nucleic acid is considered to be exogenous to a cell once introduced into the cell. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of person X is an exogenous nucleic acid with respect to a cell of person Y once that chromosome is introduced into Y's cell.

ORF (open reading frame) – An "ORF" is a series of nucleotide triplets (codons) encoding a sequence of amino acids at least 100 amino acids in length without any termination codons.

Probes and primers – Nucleic acid probes and primers may be prepared readily based on the amino acid sequences and nucleic acid sequences provided by this invention.

A **"probe"** comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and polypeptides. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed in, e.g., Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, and Ausubel *et al.* (ed.) Current Protocols in Molecular

Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987.

"Primers" are short nucleic acids, preferably DNA oligonucleotides, 10 nucleotides or more in length. A primer may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR), or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in references such as Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; Ausubel *et al.* (ed.), Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York (with periodic updates), 1987; and Innis *et al.*, PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer Designer 3 for Windows by Scientific & Educational Software (Durham, NC).

One of skill in the art will appreciate that the specificity of a particular probe or primer generally increases with the length of the probe or primer. Thus, for example, a primer comprising 20 consecutive nucleotides will anneal to a target having a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise, for example, 10, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 550, 600, 650, 700 or more consecutive nucleotides.

Recombinant – A "recombinant" nucleic acid is one having (1) a sequence that is not naturally occurring in the organism in which it is expressed or (2) a sequence made by an artificial combination of two otherwise-separated, shorter sequences. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering

techniques. "Recombinant" is also used to describe nucleic acid molecules that have been artificially manipulated, but contain the same regulatory sequences and coding regions that are found in the organism from which the nucleic acid was isolated.

Sequence identity – The similarity between two or more nucleic acid sequences or amino acid sequences is referred to as "Sequence Identity." The "percent sequence identity" between a particular nucleic acid or amino acid sequence and a sequence referenced by a particular sequence identification number is determined as follows.

First, a nucleic acid or amino acid sequence is compared to the sequence set forth in a particular sequence identification number using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained at www.fr.com or www.ncbi.nlm.nih.gov. Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to –1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastn –o c:\output.txt –q –1 –r 2.

To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastp –o c:\output.txt.

If the target sequence shares homology with any portion of the identified sequence (i.e., the sequence identified by a SEQ ID NO herein), then the designated output file will present those regions of homology as aligned sequences. If the target sequence does not share homology with any portion of the identified sequence, then the designated output file will not present aligned sequences. Once aligned, a length is determined by counting the number of consecutive nucleotides or amino acid residues from the target sequence presented in alignment with sequence from the identified sequence starting with any matched position and ending with any other matched position. A matched position is any position where an identical nucleotide or amino acid residue is presented in both the target and identified sequence. Gaps presented in the target sequence are not counted since gaps are not nucleotides or amino acid residues. Likewise, gaps presented in the identified sequence are not counted since target sequence nucleotides or amino acid residues are counted, not nucleotides or amino acid residues from the identified sequence.

The percent identity over a determined length is determined by counting the number of matched positions over that length and dividing that number by the length followed by multiplying the resulting value by 100. For example, if (1) a 1000 nucleotide target sequence is compared to the sequence set forth in SEQ ID NO: 1, (2) the Bl2seq program presents 200 nucleotides from the target sequence aligned with a region of the sequence set forth in SEQ ID NO: 1 where the first and last nucleotides of that 200 nucleotide region are matches, and (3) the number of matches over those 200 aligned nucleotides is 180, then the 1000 nucleotide target sequence contains a length of 200 and a percent identity over that length of 90 (i.e., 180 / 200 * 100 = 90).

It will be appreciated that a single nucleic acid or amino acid target sequence that aligns with an identified sequence can have many different lengths with each length having its own percent identity. For example, a target sequence containing a 20-nucleotide region (SEQ ID NO: 46) that aligns with an identified sequence (SEQ ID NO: 47) as follows has many different lengths including those listed in Table 1.

```
                          1                   20
Target Sequence:          AGGTCGTGTACTGTCAGTCA
                          | || ||| |||| |||| |
Identified Sequence:      ACGTGGTGAACTGCCAGTGA
```

TABLE 1

Starting Position	Ending Position	Length	Matched Positions	Percent Identity
1	20	20	15	75.0
1	18	18	14	77.8
1	15	15	11	73.3
6	20	15	12	80.0
6	17	12	10	83.3
6	15	10	8	80.0
8	20	13	10	76.9
8	16	9	7	77.8

It is noted that the percent identity value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It is also noted that the length value will always be an integer.

Accordingly, the invention provides nucleic acid sequences and amino acid sequences that share at least 60, 65, 70, 75, 80, 85, 90, 95, 97, and 98% sequence identity to SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22, and 23, and SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25, and 26, respectively.

Specific binding agent - A "specific binding agent" is an agent that is capable of specifically binding to the polypeptides of the present invention, and may include polyclonal antibodies, monoclonal antibodies (including humanized monoclonal antibodies) and fragments of monoclonal antibodies such as Fab, F(ab')2 and Fv fragments, as well as any other agent capable of specifically binding to the epitopes on the proteins.

Antibodies to the polypeptides, and fragments thereof, of the present invention may be useful for purification of the polypeptides. The amino acid and nucleic acid sequences provided herein allow for the production of specific antibody-based binding agents to these polypeptides.

Monoclonal or polyclonal antibodies may be produced to full-length polypeptides, polypeptides that are less than full-length, or variants thereof. Optimally, antibodies raised against epitopes on these antigens will specifically detect the polypeptides. That is, antibodies raised against the polypeptide would recognize and bind the polypeptides, and would not substantially recognize or bind to other polypeptides. The determination that an antibody specifically binds to an antigen is made by any one of a number of standard immunoassay methods; for instance, Western blotting, Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

To determine that a given antibody preparation (such as a preparation produced in a mouse against SEQ ID NO: 4) specifically detects a polypeptide having the amino acid sequence of SEQ ID NO: 4 by Western blotting, total cellular protein is extracted from cells and electrophoresed through a sodium dodecyl sulfate (SDS) polyacrylamide gel. The proteins are then transferred to a membrane (for example, nitrocellulose) and the antibody preparation is incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies can be detected with anti-mouse antibody conjugated to an enzyme such as alkaline phosphatase; application of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a densely blue-colored compound by immuno-localized alkaline phosphatase.

Isolated polypeptides suitable for use as an immunogen can be isolated from transfected cells, transformed cells, or from wild-type cells. Concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. Polypeptides that range in size from eight amino acid residues to a full-length polypeptide having enzymatic activity can be utilized as an immunogen. Polypeptides that are less than full-length may be chemically synthesized using standard methods, or may be obtained by cleavage of the whole polypeptide followed by purification of the desired size of polypeptide. Polypeptides as short as eight amino acids in length are immunogenic when presented to an immune system in the context of a Major Histocompatibility Complex (MHC) molecule, such as MHC class I or MHC class II. Accordingly, polypeptides comprising

at least 8, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 900, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350 or more consecutive (contiguous) amino acids of the disclosed amino acid sequences may be employed as immunogens for producing antibodies.

Monoclonal antibodies to any of the polypeptides disclosed herein can be prepared from murine hybridomas according to the classic method of Kohler & Milstein (Nature 256:495 (1975)) or a derivative method thereof.

Polyclonal antiserum containing antibodies to the heterogeneous epitopes of any polypeptide disclosed herein can be prepared by immunizing suitable animals with a polypeptide, which can be unmodified or modified to enhance immunogenicity. An effective immunization protocol for rabbits can be found in Vaitukaitis *et al.* (*J. Clin. Endocrinol. Metab.* 33:988-991 (1971)).

Antibody fragments can be used in place of whole antibodies and can be readily expressed in prokaryotic host cells. Methods of making and using immunologically effective portions of monoclonal antibodies, also referred to as "antibody fragments," are well known and include those described in Better & Horowitz (*Methods Enzymol.* 178:476-496 (1989)), Glockshuber *et al.* (*Biochemistry* 29:1362-1367 (1990), U.S. Pat. No. 5,648,237 ("Expression of Functional Antibody Fragments"), U.S. Pat. No. 4,946,778 ("Single Polypeptide Chain Binding Molecules"), U.S. Pat. No. 5,455,030 ("Immunotherapy Using Single Chain Polypeptide Binding Molecules"), and references cited therein.

Hybridization - "Hybridization" is a method of testing for complementarity in the base sequence of two nucleic acid molecules from different sources, and is based on the ability of complementary single-stranded DNA and /or RNA molecules to form a duplex molecule. Nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having homology to a sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22, and 23 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Once identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein.

Hybridization can be done by Southern or Northern analysis to identify a DNA or RNA sequence, respectively, that hybridizes with a nucleic acid of the invention (e.g., a probe). The probe can be labeled with a biotin, digoxygenin, an enzyme, or a radioisotope such as ^{32}P. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY. Typically, a probe is at least about 20 nucleotides in length. For example, a probe corresponding to a 20 nucleotide sequence set forth in SEQ ID NO: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22, and 23 can be used to identify an identical or similar nucleic acid. In addition, probes longer or shorter than 20 nucleotides can be used.

The invention also provides isolated nucleic acid molecules that are at least about 12 bases in length (e.g., at least about 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 100, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, or 5000 bases in length) and that hybridize, under moderate to highly stringent hybridization conditions, to the sense or antisense strand of a nucleic acid having the sequence set forth in SEQ ID NO: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22, or 23.

For the purpose of this invention, moderately stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/µg), while the washes are performed at about 50°C with a wash solution containing 2X SSC and 0.1% sodium dodecyl sulfate.

Highly stringent hybridization conditions mean the hybridization is performed at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 µg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/µg), while the washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% sodium dodecyl sulfate.

Sequence Variants- With the provision of the amino acid sequences set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25, and 26 and the corresponding nucleic acid sequences set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22, and 23, variants of these sequences can be created. The sequence of these variants share from about 50% to about 99% sequence identity with the corresponding sequence provided in the accompanying sequence listing. In other embodiments, the variants share at least 55, 60, 65, 70, 75, 80, 85, 87, 90, 92, 94, 96, or 98% sequence identity with the sequences described herein.

Variant polypeptides sequences include polypeptides that differ in amino acid sequence from the polypeptides sequences disclosed, but that retain biological activity (e.g., enzymatic activity). Such polypeptides may be produced by manipulating the nucleotide sequence encoding the enzyme using standard procedures such as site-directed mutagenesis or the polymerase chain reaction. The simplest modifications involve the substitution of one or more amino acids for amino acids having similar biochemical properties. These so-called "conservative substitutions" are likely to have minimal impact on the activity of the resultant polypeptide. Table 2 provides examples of conservative substitutions.

TABLE 2

Original Residue	Conservative Substitution(s)
Arg	Lys
Asn	Gln
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; His
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe

Val	Ile; Leu

More substantial changes in enzymatic function or other features may be obtained by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining: (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation; (b) the charge or hydrophobicity of the molecule at the target site; or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in protein properties will be those in which: (a) a hydrophilic residue, e.g., serine or threonine, is substituted for a hydrophobic residue, e.g., leucine, isoleucine, phenylalanine, valine or alanine, or vice versa; (b) a cysteine or proline is substituted for any other residue; (c) a residue having an electropositive side chain, e.g., lysine, arginine, or histidine, is substituted for an electronegative residue, e.g., glutamine or aspartamine, or vice versa; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for one not having a side chain, e.g., glycine, or vice versa. The effects of these amino acid substitutions, deletions, or additions can be assessed for polypeptides having enzyme activity by analyzing the ability of the polypeptide to catalyze the conversion of the same substrate as the related native polypeptide to the same product as the related native polypeptide. Accordingly, polypeptide having 5, 10, 20, 30, 40, 50 or less conservative amino acid substitutions are provided by the invention.

Polypeptides and nucleic acids encoding polypeptides can be produced by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (ed.), Molecular Cloning: A Laboratory Manual 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, N.Y., 1989, Ch. 15. By the use of such techniques, variants may be created that differ in minor ways from the native sequence, yet that still encode a polypeptide having enzymatic activity. In their simplest form, such variants may differ from the disclosed sequences by alteration of the coding region to fit the codon usage bias of the particular organism into which the molecule is to be introduced.

Alternatively, the coding region may be altered by taking advantage of the degeneracy of the genetic code to alter the coding sequence in such a way that, while the nucleotide sequence is substantially altered, it nevertheless encodes a protein having, an

amino acid sequence identical or substantially similar to the disclosed polypeptide sequences. For example, the 5th amino acid residue of the SEQ ID NO: 18 is alanine. This is encoded in the open reading frame (ORF) by the nucleotide codon triplet GCG. Because of the degeneracy of the genetic code, three other nucleotide codon triplets-- GCA, GCC, and GCT --also code for alanine. Thus, the nucleotide sequence of the ORF can be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the cDNA and gene sequences disclosed herein using a standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. Thus, this invention also encompasses nucleic acid sequences that encode the polypeptides but that vary from the disclosed nucleic acid sequences by virtue of the degeneracy of the genetic code.

Transformed – A "transformed" cell is a cell into which a nucleic acid molecule has been introduced by molecular biology techniques. As used herein, the term "transformation" encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including, but not restricted to, transfection with a viral vector, conjugation, transformation with a plasmid vector, and introduction of naked DNA by electroporation, lipofection, particle gun acceleration.

Nucleic Acid Constructs - Polypeptides of the invention can be produced by ligating a nucleic acid molecule encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic production cell with the expression vector. In general, nucleic acid constructs include expression control elements operably linked to a nucleic acid sequence encoding a polypeptide of the invention (e.g., lycopene ε cyclase transferase A, B, or C). Expression control elements do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the expression control elements to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Expression control elements can include, for example, promoter sequences, enhancer sequences, response elements, polyadenylation sites, or inducible elements.

In bacterial systems, a strain of *E. coli* such as DH10B or BL-21 can be used. Suitable *E. coli* vectors include, but are not limited to, pUC18, pUC19, the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST), and pBluescript series of vectors. Transformed *E. coli* are typically grown exponentially then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, fusion proteins produced from the pGEX series of vectors are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express polypeptides of the invention. A nucleic acid encoding a polypeptide of the invention can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing polypeptides of the invention can be identified by standard methodology. Alternatively, a nucleic acid encoding a polypeptide of the invention can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

A polypeptide within the scope of the invention can be "engineered" to contain an amino acid sequence that allows the polypeptide to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ tag (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino termini. Other fusions that could be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase.

Agrobacterium-mediated transformation, electroporation and particle gun transformation can be used to transform plant cells. Illustrative examples of transformation techniques are described in U.S. Patent No. 5,204,253 (particle gun) and U.S. Patent No. 5,188,958 (*Agrobacterium*). Transformation methods utilizing the Ti and Ri plasmids of *Agrobacterium spp.* typically use binary type vectors. Walkerpeach, C. et

al., in Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994). If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art.

Production Cell – a cell that can be cultured such that it produces the carotenoids described herein and/or the polypeptides and nucleic acid sequences described herein. This includes, without limitation, prokaryotic cells such as *R. sphaeroides* cells and eukaryotic cells such as plant, yeast, and other fungal cells. It is noted that cells containing an isolated nucleic acid of the invention are not required to express the isolated nucleic acid. In addition, the isolated nucleic acid can be integrated into the genome of the cell or maintained in an episomal state. In other words, cells can be stably or transiently transfected with an isolated nucleic acid of the invention.

Any method can be used to introduce an isolated nucleic acid into a cell. In fact, many methods for introducing nucleic acid into a cell, whether *in vivo* or *in vitro*, are well known to those skilled in the art. For example, calcium phosphate precipitation, conjugation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer are common methods that can be used to introduce nucleic acid molecules into a cell. In addition, naked DNA can be delivered directly to cells *in vivo* as describe elsewhere (U.S. Pat. Nos. 5,580,859 and 5,589,466). Furthermore, nucleic acid can be introduced into cells by generating transgenic animals.

Any method can be used to identify cells that contain an isolated nucleic acid within the scope of the invention. For example, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis can be used. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a cell contains a particular nucleic acid by detecting the expression of a polypeptide encoded by that particular nucleic acid. For example, the polypeptide of interest can be detected with an antibody having specific binding affinity for that polypeptide, which indicates that that cell not only contains the introduced nucleic acid but also expresses the encoded polypeptide. Enzymatic activities of the polypeptide of interest also can be detected or an end product (e.g., a particular carotenoid) can be detected as an indication that the cell contains the introduced nucleic acid and expresses the encoded polypeptide from that

introduced nucleic acid.

The cells described herein can contain a single copy, or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid. For example, a bacterial cell (e.g., *Rhodobacter*) can contain about 50 copies of an exogenous nucleic acid of the invention. In addition, the cells described herein can contain more than one particular exogenous nucleic acid. For example, a bacterial cell can contain about 50 copies of exogenous nucleic acid X as well as about 75 copies of exogenous nucleic acid Y. In these cases, each different nucleic acid can encode a different polypeptide having its own unique enzymatic activity. For example, a bacterial cell can contain two different exogenous nucleic acids such that a high level of a carotenoid is produced. In addition, a single exogenous nucleic acid can encode one or more polypeptides. For example, a single nucleic acid can contain sequences that encode three or more different polypeptides.

Microorganisms that are suitable for producing carotenoids may or may not naturally produce carotenoids, and include prokaryotic and eukaryotic microorganisms, such as bacteria, yeast, and fungi. In particular, yeast such as *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*), *Candida utilis*, and *Saccharomyces cerevisiae*, fungi such as *Neurospora crassa*, *Phycomyces blakesleeanus*, *Blakeslea trispora*, and *Aspergillus sp*, Archaea bacteria such as *Halobacterium salinarium*, and Eubacteria including *Pantoea* species (formerly called *Erwinia*) such as *Pantoea stewartii* (e.g., ATCC Accession #8200), flavobacteria species such as *Xanthobacter autotrophicus* and *Flavobacterium multivorum*, *Zymonomonas mobilis*, *Rhodobacter* species such as *R. sphaeroides* and *R. capsulatus*, *E. coli*, and *E. vulneris* can be used. Other examples of bacteria that may be used include bacteria in the genus *Sphingomonas* and Gram negative bacteria in the α-subdivision, including, for example, *Paracoccus*, *Azotobacter*, *Agrobacterium*, and *Erythrobacter*. Eubacteria, and especially *R. sphaeroides* and *R. capsulatus*, are particularly useful. *R. sphaeroides* and *R. capsulatus* naturally produce certain carotenoids and grows on defined media. Such *Rhodobacter* species also are non-pyrogenic, minimizing health concerns about use in nutritional supplements. *Streptomyces aeriouvifer*, *Bacillus subtilis*, and *Staphylococcus aureus* also are suitable production cells. In some embodiments, it can be useful to produce carotenoids in plants

and algae such as *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella protothecoides*, *Zea mays*, *Brassica napus*, *Arabidopsis thaliana*, *Tagetes erecta*, *Lycopersicum esculentum*, and *Neospongiococcum excentrum*.

It is noted that bacteria can be membranous or non-membranous bacteria. The term "membranous bacteria" as used herein refers to any naturally-occurring, genetically modified, or environmentally modified bacteria having an intracytoplasmic membrane. An intracytoplasmic membrane can be organized in a variety of ways including, without limitation, vesicles, tubules, thylakoid-like membrane sacs, and highly organized membrane stacks. Any method can be used to analyze bacteria for the presence of intracytoplasmic membranes including, without limitation, electron microscopy, light microscopy, and density gradients. See, e.g., Chory et al., (1984) J. Bacteriol., 159:540-554; Niederman and Gibson, Isolation and Physiochemical Properties of Membranes from Purple Photosynthetic Bacteria. In: The Photosynthetic Bacteria, Ed. By Roderick K. Clayton and William R. Sistrom, Plenum Press, pp. 79-118 (1978); and Lueking et al., (1978) J. Biol. Chem., 253: 451-457.

Examples of membranous bacteria that can be used include, without limitation, Purple Non-Sulfur Bacteria, including bacteria of the Rhodospirillaceae family such as those in the genus *Rhodobacter* (e.g., *R. sphaeroides* and *R. capsulatus*), the genus *Rhodospirillum*, the genus *Rhodopseudomonas*, the genus *Rhodomicrobium*, and the genus *Rhodopila*. The term "non-membranous bacteria" refers to any bacteria lacking intracytoplasmic membrane. Membranous bacteria can be highly membranous bacteria. The term "highly membranous bacteria" as used herein refers to any bacterium having more intracytoplasmic membrane than *R. sphaeroides* (ATCC 17023) cells have after the *R. sphaeroides* (ATCC 17023) cells have been (1) cultured chemoheterotrophically under aerobic condition for four days, (2) cultured chemoheterotrophically under anaerobic for four hours, and (3) harvested. Aerobic culture conditions include culturing the cells in the dark at 30°C in the presence of 25% oxygen. Anaerobic culture conditions include culturing the cells in the light at 30°C in the presence of 2% oxygen. After the four hour anaerobic culturing step, the *R. sphaeroides* (ATCC 17023) cells are harvested by centrifugation and analyzed.

II. Brief Overview

The present invention involves the identification, isolation, and cloning of genes involved in a non-mevalonate pathway for carotenoid biosynthesis. In particular, the isolated genes allow for the biosynthesis of a C40 carotenoid and the conversion of the C40 carotenoid to a C50 carotenoid. The isolated genes can be introduced into a production cell. The production cell can be used to produce the polypeptides for use *in vitro* (outside of the cell) or the production cell can be used to make C>40 carotenoids, such as C50 carotenoids and various derivatives.

The identification of one set of representative genes allows for the isolation of genes that have similar nucleic acid and/or amino acid sequences, which have a similar function. The isolated genes offer an advance in the art, because they allow for the conversion of a C40 carotenoid to a C>40 carotenoid, such as a C50 carotenoid.

The nucleic acid sequences provided herein encode three separate polypeptides. An important finding of the invention is that the activity of all three polypeptides can be used to convert a C40 carotenoid to the C50 carotenoid. The nucleic acid molecules were first isolated from *A. mediolanus*. Similar genes with substantial homology were then isolated from *M. luteus*. The genes from *M. luteus* were also shown to be active. It is believed that other similar genes with substantial homology could be isolated from other bacteria using similar techniques, and that such genes fall within the present invention.

The present invention is particularly important because it provides a key step to the ability to convert carotenoids from the C40 level to the C50 level by genetic manipulation.

The invention uses standard laboratory practices, such as for the cloning, manipulation, and sequencing of nucleic acids, purification and analysis of proteins and other molecular biological and biochemical techniques, unless otherwise specified. Such standard techniques are explained in detail in standard laboratory manuals such as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd edition., vol. 1-3, Cold Spring Harbor, New York, 1989; and Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publ. Assoc. & Wiley-Intersciences, 1989.

III. Experimental Materials, Methods, Results, and Examples—*Agromyces mediolanus*

Brief outline of the subject matter described in section III

1. The selection of *A. mediolanus* as the bacterium for which genomic DNA would be extracted.

2. The construction of a genomic DNA library, the isolation of genomic colonies, and the selection of experimental working colonies. A particularly important experimental working colony was called Y1.

3. The isolation of a plasmid DNA from the Y1 colony, and the identification of a carotenogenic operon contained therein.

4. The sequencing and sequence analysis of the carotenogenic operon.

5. The identification of seven (7) genes (*idi*, *crtE*, *crtB*, *crtI*, *lctA* (ORF X1), *lctB* (ORF X2), and *lctC* (ORF Y) from the operon, wherein one or more of the seven (7) isolated genes allow for the biosynthesis of the C50 carotenoid and the conversion of a C40 carotenoid to a C>40 carotenoid, such as a C50 carotenoid. The identification included, among other aspects, the determination of the respective nucleic acid sequences and encoded amino acid sequences.

6. The creation of constructs of certain combinations of the seven genes. The constructs were amplified with primers and PCR. Deductive analysis was performed on the amplified constructs to determine the capabilities of individual constructs. The pathway of the associated biosynthetic reactions was determined. The portion of the pathway associated with individual genes was also determined.

7. The recognition that four of the previously unidentified genes (4) (*idi*, *crtE*, *crtB*, *crtI*) of the seven (7) isolated genes allow for the production of a C40 carotenoid, in a manner having certain similarities to techniques already known it the art.

8. The realization that three (3) (*lctA*, *lctB*, *lctC*) of the seven (7) isolated genes represented a significant advance to the art, because the genes allow for the conversion of a C40 carotenoid to a C>40 carotenoid, such as a C50 carotenoid.

9. The realization that the activities that are provided by the three (3) genes (*lctA*, *lctB*, *lctC*) can be used to convert a C40 carotenoid to a C50 carotenoid in a single step.

10. The cloning of certain constructs of the seven (7) isolated genes into host bacteria, which resulted in successful carotenogenic reactions.

Details elaborating the brief outline are described in the remainder of section III.

A. Selection of *Agromyces mediolanus; Agromyces mediolanus* genomic DNA preparation

Flavobacterium dehydrogenans was chosen as the bacterial source for the identification of genes since the bacterium had been reported to produce both C40 and C50 carotenoids (Weeks OB *et al. Nature* **224**:879-82, 1969). Since *F. dehydrogenans* was an unidentified bacterium in the ATCC (American Type Culture Collection), the strain was submitted for identification. Microbial identification revealed the organism to be *Agromyces mediolanus*. Although there were reports in the literature describing the production of the C50 carotenoid decaprenoxanthin in (*F. dehydrogenans*) *A. mediolanus* (Schwieter U, and Liaaen-Jensen S. *Acta Chem Scand* **23**:1057, 1969, and Liaaen-Jensen S, *et al. Acta Chem Scand 22:*1171-86, 1968), no reports were found on the genes responsible for C50 carotenoid biosynthesis.

A. mediolanus was grown in 200 mL of nutrient broth for 36 hours at 30°C and 250 rpm. Cultured cells were centrifuged to form a cell pellet, and washed by resuspending the pellet in a 10 mM Tris:1 mM EDTA (ethylene diaminetetraacetate) solution, and centrifuged again. The cell pellets were resuspended in 5 mL of GTE buffer (50 mM glucose, 25 mM Tris HCl, pH 8.0, 10 mM EDTA, pH 8.0) per 100 mL of culture. The bacterial cell walls were lysed by adding lysozyme and Proteinase K, each to a 1.0 mg/mL final concentration, and mutanolysin to a 5.5 μg/mL final concentration. After a 1.5 hours incubation at 37°C, SDS (sodium dodecyl sulfate) was added to a final concentration of 1% and the concentration of Proteinase K was brought to 2 mg/mL. After incubation at 50°C for one hour, the solution containing the lysed cells was diluted 1:1 with fresh GTE buffer and NaCl was added to a 0.15 M concentration in the diluted solution. The mixture was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 x g for 10 minutes. The supernatant was removed and placed in a clean tube, extracted with an equal volume of chloroform, and centrifuged at 3,000 x g for 10 minutes. The supernatant was treated with RNase and

precipitated with 2.5 volumes of ethanol. After mixing the solution, the precipitated DNA was removed by spooling it on a glass rod. The spooled DNA was washed with 70% ethanol, air dried, and resuspended in 10 mM Tris, pH 8.5.

B. *A. mediolanus* genomic DNA library construction for isolation of the carotenoid operon

A. mediolanus genomic DNA (80 μg) was digested at 37°C for 10 minutes with 2.8 units of *Sau3A* I restriction enzyme (Promega, Madison, WI). The digested DNA was separated by gel electrophoresis using a 0.8% Tris-acetate-EDTA (TAE) agarose gel. DNA fragments ranging from 7-10 Kb in size were excised and purified using a Qiagen Gel Purification kit (Qiagen Inc., Valencia, CA). Vector to be used in the ligation (pUC19) was prepared by digesting with *BamH* I restriction enzyme (New England Biolabs, Inc., Beverly, MA), gel purifying, and dephosphorylating using shrimp alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN). *BamH* I DNA fragments (126 ng) were ligated into 50 ng of prepared pUC19 DNA at 14°C for 16 hours using T4 DNA ligase (Roche Molecular Biochemicals). The ligation reaction was precipitated by adding 1/10 volume 7.5 M NH_4OAc and 2.5 volumes ethanol, incubating at -20°C for 3 hours, centrifuging to obtain a DNA pellet, washing the pellet with 70% ethanol, drying the pellet, and resuspending the pellet in 20 μL of 10 mM Tris buffer, pH 8.5. One microliter of ligation reaction was used to electroporate 40 μL of ElectroMAX™ DH10B™ competent cells (Life Technologies, Inc., Rockville, MD). Electroporated cells were recovered in SOC media and plated on LB plates containing 100 μg/mL of ampicillin (LBA). The plating volume necessary to produce approximately 300 cells/plate was determined by plating various volumes of transformed cells. Using this information, 125 plates containing approximately 300 colonies each were plated from transformations using remains of the ligation reaction. Plates were incubated at 37°C for one day and then at room temperature for one day. On the second day, one yellow colony (Y1) was identified and streaked to a new LBA plate. Plasmid DNA of this colony was isolated using a Qiaprep Spin Miniprep Kit (Qiagen, Inc.). *EcoR* I restriction digests (New England Biolabs, Inc.) of the plasmid DNA showed the plasmid to contain an insert approximately 9-Kb in size.

C. Subcloning and sequencing of the *A. mediolanus* carotenogenic operon

Several restriction enzymes, including *BamH* I and *Pst* I, were used to digest 2 µg aliquots of plasmid DNA from the Y1 colony. A digest from *BamH* I produced two fragments approximately 9 Kb and 3 Kb in size and a digest from *Pst* I produced four fragments approximately 4.5, 3.0, 1.5, and 1.0 Kb in size. These fragments were gel purified, ligated into pUC19, and transformed into ElectroMAX™ DH10B™ competent cells as described above. The electroporated cells were plated on LB agar plates with 100 µg/mL of ampicillin and 50 µg/mLof 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (Xgal, media =LBAX). Single, white colonies corresponding to each purified fragment were isolated. Plasmid DNA was isolated and used to obtain the DNA sequence of each insert, using either M13F and M13R vector primers or sequencing primers designed from internal DNA sequence. Individual sequences were aligned using the software Clone Manager and Align Plus (Scientific and Educational Software, Durham, NC).

D. Sequence analysis of the *A. mediolanus* carotenogenic operon

The BLAST DNA sequence comparison program (National Center for Biotechnology Information) was used to identify genes residing on the insert of the Y1 clone. The sequence of nucleotides residing on the insert of the Y1 clone was chosen as a working operon (the Y1 operon), and the location of the genes residing on the Y1 operon is shown in FIG 1. The BLAST analysis identified the following genes, in order of location in the operon:

* *idi*, isopentenyl pyrophosphate isomerase,
* *crtE*, geranylgeranyl pyrophosphate synthase (CCPS synthese),
* *crtB*, phytoene synthase, and
* *crtI*, phytoene dehydrogenase (phytoene desaturase).

In addition, three open reading frames (ORFs) downstream of *crtI* were identified to which no definitive function could be assigned using sequence similarity. The three ORFs were given the following names:

* ORFX1—the first ORF downstream of *crtI*—was 372 nucleotides in length
* ORFX2—the second ORF downstream of *crtI*—was 348 nucleotides in length
* ORFY—the third ORF downstream of *crtI*—was 897 nucleotide in length

ORFX1 showed homology (33% sequence identity) to the lycopene cyclase domain of the *Rhizomucor carRP* gene. The *carRP* gene encodes a polypeptide having both phytoene synthase and lycopene cyclase activities. Therefore, it is likely that the polypeptide encoded by the ORFX1 gene contributes cyclase activity during the conversion of lycopene to decaprenoxanthin.

No genes with significant homology were detected for ORFX2 in the Genbank database. The ORFY protein sequence had low homology with a DHNA-octaprenyltransferase from *Bacillus subtilis* in the Swisspro database. This enzyme catalyzes the attachment of a 40-carbon side chain to 1,4-dihydroxy-2-naphthoic acid (DHNA). BLAST searches of the ORFY DNA sequence to the NCBI non-redundant DNA database showed certain homology to ORFs identified in *Deinococcus radiodurans, Halobacterium* sp. NRC-1 (National Research Council of Canada, a cell repository), and *Methanobacterium thermoautotrophicum*. The *Deinococcus radiodurans* ORF in turn shows low homology to a *Schizosaccharomyces pombe* para-hydroxybenzoate polyprenyltransferase. The *Halobacterium* ORF shows significant homology to a *Rhodobacter capsulatus* bacteriochlorophyll synthase gene, which catalyzes the esterification of bacteriochlorophyll by geranylgeranyl-pyrophosphate, and low homology to a *Saccharomyces cerevisiae* para-hydroxybenzoate polyprenyltransferase.

E. *A. mediolanus* DNA constructs for carotenoid production

1. The constructs and carotenoid production

Initial data indicated that the inclusion of the *idi* gene in an expression vector was likely necessary to achieve detectable carotenoid expression levels. The initial experiments also indicated that the use of a medium copy number vector was preferable to use of a high copy number vector, possibly due to a detrimental effect on the bacterial cell of maintaining the latter. Therefore, the expression vector pProLarNde was used. This vector is a modification of the pPROLar.A vector (CLONTECH Laboratories, Inc., Palo Alto, CA) into which an *Nde* I restriction site was inserted downstream of the ribosomal binding site.

Primers were designed to amplify three regions of the Y1 operon: (a) the region from *idi* through *crtI*—the *idi-crtI* construct (4.6 KB), (b) the region from *idi* through ORFX2—the *idi*-ORFX2 construct (5.3 KB), and (c) the region from *idi* through ORFY—the *idi*-ORFY construct (6.7 Kb). These primers were designed to introduce an *Nde* I restriction site at the beginning of the amplified fragment and a Hind III restriction site at the end of the amplified fragment. The sequences of the primers were as follows, with the restriction sites underlined:

Primer name	Primer sequence
AIDINDEF	5'-TTCATATGTCACTAGCCAGGCGAGATATCC-3' (SEQ ID NO: 27)
APDHIIIR	5'-GAAAGCTTAAGAAGATGCCGAGCGAGATG-3' (SEQ ID NO: 28)
AXHIIIR	5'-AGAAGCTTTGTACGGCACGAGGAAGAACAG-3' (SEQ ID NO: 29)
AYHIIIR	5'-GAAAGCTTCTCCGTGACGAGATCCTGAG-3' (SEQ ID NO: 30)

Due to the high GC content of *A. mediolanus*, PCR was conducted using the Advantage®–GC Genomic Polymerase (CLONTECH) kit. The PCR reaction mix, according to manufacturer's specifications, used a 1.0 M final GC-Melt concentration and 1.0 ng of *A. mediolanus* genomic DNA per μL of reaction mix in a 100-200 μL reaction. The PCR reactions were performed in a Perkin Elmer Geneamp system 2400 under the following conditions: (a) an initial denaturation at 94°C for 45 seconds; (b) 8 cycles of (1) 94°C for 25 seconds, (2) 56°C for 1 minute, and (3) 72°C for 10 minutes; (c) 25

cycles of (1) 94°C for 25 seconds, (2) 60°C for 1 minute, and (3) 72°C for 10 minutes; and (d) a final extension of 72°C for 10 minutes. The PCR reactions were subjected to gel electrophoresis using a 0.8 % TAE agarose gel. Fragments of the expected sizes were gel purified as previously described. Purified DNA was digested overnight with Hind III and *Nde* I to make the fragment ends compatible with digested pPROLarNde vector. The digested PCR product was purified using a Qiagen PCR Purification column and quantified on a spectrophotometer.

pPROLarNde vector (5 μg) was digested overnight with Hind III and *Nde* I and purified using gel electrophoresis on a 1% TAE agarose gel and a Qiagen Gel Purification Kit. The digested and purified vector was dephosphorylated using calf intestinal alkaline phosphatase (CIAP, Promega) according to manufacturer's specifications with the following exceptions: (a) 40 μL of eluent from the Qiagen purification was used directly as the starting DNA, (b) the CIAP was used at a 1/20 enzyme dilution rather than a 1/100 dilution, and (c) the dephosphorylated DNA was purified using a Qiagen PCR Purification Column rather than by ethanol precipitation.

The purified and digested PCR products were each ligated into 50 ng of prepared pPROLarNde DNA at 16°C for 16 hours using T4 DNA ligase (Roche Molecular Biochemicals). One μL of each ligation reaction was used to electroporate 40 μL of ElectroMAX™ DH10B™ competent cells. Electroporated cells were recovered in SOC media for one hour and plated on LB plates containing 50 μg/mL of kanamycin, 1 mM isopropylthio-β-D-galactoside (IPTG), and 2% L-arabinose (LBKIA).

Two red colonies were isolated from *E. coli* transformed with the *idi-crtI* construct; two red colonies were isolated from *E. coli* transformed with the *idi*-ORFX2 construct; one yellow colony was isolated from *E. coli* transformed with the *idi*-ORFY construct. Each of these colonies had the desired insert size, as indicated by PCR and by restriction enzyme digest with Hind III and *Nde* I. DNA sequencing of the X1-X2-Y region was conducted on plasmid DNA from these colonies to check for PCR errors.

Carotenoids were extracted from 100 mL cultures grown for 3 days in LBKIA media at 30°C and 200 rpm. Cells were pelleted by centrifugation at 12,000 g for 10 minutes, washed with sterile distilled water, and re-centrifuged. The pellet was dried and resuspended in 2 mL of acetone by vortexing in the presence of glass beads. The

extraction of the carotenoids was performed at 55°C for a total of 1.5 hours and at room temperature for one hour. Extractions were conducted in the dark to prevent light-induced degradation of carotenoids, and with vortexing every 15 minutes to enhance cell exposure to the solvent. The extraction mixture was then centrifuged at 27,00 g for 15 minutes to obtain a hard pellet of cell matter. The supernatant of the carotenoids was passed through a 0.2 micron filter and the absorption curve from 400–600 nm was read on a Cary 100 spectrophotometer.

HPLC analysis of the carotenoid extracts from various clones is shown in FIG 2 and FIG 3. It is significant that the C50 carotenoid extracted from the *E. coli* clone with the *idi-Y A. mediolanus* fragment showed a mass that was identical to that observed in *A. mediolanus* wild type extract (FIG 4). Absorption curves showed that the carotenoid material produced from *E. coli* containing the *idi-crtI* construct and the carotenoid material produced from *E. coli* containing the *idi*-ORFX2 construct have a spectrum identical to that of lycopene (a C40 carotenoid) (FIG 5). HPLC analysis of the extracts and mass spectrometric analysis confirmed these observations (FIG 7).

The carotenoid material produced from the *idi*-ORFY construct exhibited a spectrum that appeared to be a mixture of carotenoids, including both lycopene (FIG 6) and the C50 carotenoid produced by the original Y1 clone (FIG 3B).

2. The relationship of ORFX1, ORFX2, and ORFY to the production of the C50 carotenoid

The production of the C50 carotenoid by the *E. coli* clone having the *idi*-ORFY construct and lack of production by the clone having the *idi*-ORFX2 construct indicate that ORFY was necessary for production of the Y1 C50 carotenoid. To help determine whether the X1 and X2 ORFs were also necessary for production of the C50 carotenoid, the following strategies were employed:

The first strategy is detailed in Example 1, and it involved cloning ORFY into the *idi-crtI*/pPROLarNde construct to determine if the C50 carotenoid could be produced in the absence of the X1 and X2 ORFs. Primers for the amplification of ORFY were designed to introduce a *Pac* I restriction site at the beginning of the amplified fragment and an *Xba* I restriction site at the end of the amplified fragment, which would insert the

ORFY fragment downstream of the *idi-crtI* genes. The sequences of the primers were as follows, with the restriction sites underlined:

AYPACF 5'-GTCTTAATTAACTGCTGCTCTGCTCCACGGTCT -3' (SEQ ID NO: 31)
AYXBAR 5'-TATCTAGACGCTCCGTGACGAGATCCTGAG -3' (SEQ ID NO: 32)

The PCR reaction mix contained 1X *Pfu* buffer, 0.2 mM each dNTP, 5% dimethyl sulfoxide (DMSO), 0.5 μM each primer, 10 units of *Pfu* DNA polymerase (Stratagene) and 200 ng of *A. mediolanus* genomic DNA in a 200 μL reaction. The PCR reactions were performed in a Perkin Elmer Geneamp system 2400 under the following conditions: an initial denaturation at 94°C for 1 minute, 8 cycles of (1) 94°C for 30 seconds, (2) 57°C for 45 seconds, and (3) 72°C for 3.5 minutes; 25 cycles of (1) 94°C for 30 seconds, (2) 62°C for 45 seconds, and (3) 72°C for 3.5 minutes; and a final extension of 72°C for 7 minutes. The PCR reactions were subjected to gel electrophoresis using a 1.0 % TAE agarose gel. A fragment of the expected size was gel purified as previously described. Purified DNA was digested overnight with *Pac* I, purified using a Qiagen PCR purification column, digested for 3.5 hours with *Nde* I restriction enzyme, purified with a Qiagen PCR purification column, and eluted in 30 μL of 10 mM Tris.

The *idi-crtI* construct was similarly digested with *Pac* I and *Xba* I, dephosphorylated with shrimp alkaline phosphatase (Roche, Basil, Switzerland), and gel purified. Eighty μg of the digested and purified *idi-crtI* construct was ligated with 120 ng of the ORFY product using T4 DNA ligase at 16°C for 16 hours. A control ligation with no insert DNA was also performed. One microliter of each ligation reaction was used to transform *E. Coli* ElectroMAX™ DH10B™ competent cells. The transformation reactions were recovered in 300 μL of SOC media for 1 hour and plated on both LB media with 50 μg/mL kanamycin (LBK) and LBKIA media. Several colonies that grew on the LBK plates were patched to LBKIA plates. Plasmid DNA was isolated from single colonies and shown to have the desired insert size through digestion with *Xba* I restriction enzyme.

The second strategy used a two-vector system. ORFY was cloned into the *Sph I/Xba* I sites of pUC19 and used in double transformations with the *idi-*

crtI/pPROLarNde vector. Plasmid DNA was isolated from single colonies and digested with *Xba* I and an *Xba* I/*Sph* I mix to check the insert size. Electrocompetent cells of *E. coli* strain DH5αPRO (CLONTECH) were transformed with both the *idi-crtI*/pPROLarNde vector and the ORFY/pUC19 vector in a 5:1 ratio due to a lower transformation rate of the first vector. Cells were recovered in SOC media for 1 hour and plated on LB media containing 100 μg/mL ampicillin and 50 μg/Ml kanamycin (LBAK) and LBKIA media with 100 μg/mL ampicillin (LBAKIA). Single colonies were patched to new LBAKIA plates. All resulting colonies were red in color. Plasmid DNA was isolated from double transformants and digested with *Xba* I to check the size of both plasmids. Carotenoids were extracted from the clones and identified as lycopene (a C40 carotenoid) on the basis of the visible spectral profile.

The experiments described in the first and second strategies indicate that the *idi*-crtI construct with the addition of ORF Y—but without ORFX1 and ORFX2—can produce C40 carotenoids but did not produce C50 carotenoids.

The third strategy is detailed in Example 3 and involves site-directed mutagenesis to introduce frameshift mutations individually in ORFX1, ORFX2, and ORFY to help determine if the X1 and X2 ORFs were needed for production of the Y1 C50 carotenoid. A plasmid containing the X1, X2, and Y ORFs in pUC19 was constructed as follows and used as template for mutagenic PCR. The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was then used to produce a vector containing a mutation in ORFX1, a vector with a mutation in ORFX2, and a vector containing a mutation in ORFY. Primers were designed to amplify the region of *A. mediolanus* genomic DNA containing the X1, X2, and Y ORFs. These primers were designed to introduce an *Sph* I restriction site at the beginning of the amplified fragment and an *Xba* I restriction site at the end of the amplified fragment. The sequences of the primers were as follows, with the restriction sites underlined:

AXSPHF 5'-TAGGCATGCAACGTCGAGGGGCTGTACTTC -3' (SEQ ID NO: 33)
AYXBAR 5'-TATCTAGACGCTCCGTGACGAGATCCTGAG -3' (SEQ ID NO: 32)

As part of the third strategy, the non-mutated ORFX1, ORFX2, ORFY fragment was combined with an *idi-crtI* fragment. This was done using PCR conducted using the Advantage®–GC Genomic Polymerase (CLONTECH) Kit. The PCR reaction mix was according to manufacturer's specifications, using a 1.0 M final GC-Melt concentration and 1.0 ng of *A. mediolanus* genomic DNA per µl of reaction mix in a 100-200 µL reaction. The PCR reactions were performed in a Perkin Elmer Geneamp system 2400 under the following conditions: an initial denaturation at 94°C for 1 minute, 8 cycles of (1) 94°C for 30 seconds, (2) 56°C for 45 seconds, and (3) 72°C for 3.75 minutes; 25 cycles of (1) 94°C for 30 seconds, (2) 60°C for 45 seconds, and (3) 72°C for 3.75 minutes; and a final extension of 72°C for 7 minutes. The PCR reactions were subjected to gel electrophoresis using a 1.0 % TAE agarose gel. Fragments of the expected size were gel purified as previously described. Purified DNA was digested overnight with *Xba* I and *Sph* I restriction enzymes to make the fragment ends compatible with digested vector and purified using a Qiagen PCR Purification column.

The pUC19 vector was digested with *Sph* I and *Xba* I, gel purified, and dephosphorylated as described previously. The digested and purified vector (65 ng) was ligated with 360 ng of the X1X2Y insert using T4 DNA ligase at 16°C for 16 hours. A control ligation with no insert DNA was also performed. One microliter of each ligation reaction was used to transform *E. coli* ElectroMAX™ DH10B™ competent cells. The transformation reaction was recovered in 300 µL of SOC media for 1 hour and plated on LBAX media. Single, white colonies were screened by PCR to determine if they contained the desired insert. Plasmid DNA was isolated from seven colonies positive for the insert. Equal amounts of DNA of each of the seven plasmids was pooled. 25 ng of the pooled X1X2Y/pUC19 plasmid DNA and 100 ng of *idi-crtI* plasmid DNA were transformed into electrocompetent cells of the *E. coli* strain DH5αPRO. Cells were recovered for 1 hour in SOC media and plated on LBAK and LBAKIA media. The resulting colonies were either yellow or red, with red colonies presumably resulting from errors in DNA replication during PCR of the X1X2Y fragment. Plasmid DNA was isolated for three yellow colonies and exhibited the desired inserts upon digestion with *Xba* I. Carotenoid extractions on these three cultures showed that they were producing the C50 carotenoid of the original Y1 clone. Thus, the non-mutated ORFX1, ORFX2,

ORFY fragment combined with the *idi-crtI* fragment was capable of producing a C50 carotenoid when introduced into *E. coli*.

As another part of the third strategy, mutated ORFX1, ORFX2, and ORFY fragments were individually combined with an *idi-crtI* fragment.

The following primers were used in mutagenesis:

X1A 5'-GCTCGTCGACGCGCGCTAGCCGGCTGTTCTTCTGG -3' (SEQ ID NO: 34)

X1B 5'-CCAGAAGAACAGCCGGCTAGCGCGCGTCGACGAGC -3' (SEQ ID NO: 35)

The underlined base was inserted, causing a frameshift mutation and creating a unique *Nhe* I site in the plasmid.

In addition, a C nucleotide and a G nucleotide were deleted, respectively, from the spaces in the X2A primer and a C nucleotide and a G nucleotide were deleted, respectively, from the spaces in the X2B primer. The first mutation introduced a frameshift and a unique *Nhe* I site, while the second mutation eliminated a potential translational start codon.

X2A 5'-GGAACGGGAGGCAGAGCA GGC TAGCTCATCGGCGGGCCCTTCG-3' (SEQ ID NO: 36)

X2B 5'-GGGCCCGCCGATGAGCTA GCC TGCTCTGCCTCCCGTTCC-3' (SEQ ID NO: 37)

A G nucleotide was deleted from the space in the YA primer and a C was deleted from the space in the YB primer, in order to create a frameshift and a unique *Nhe* I site.

YA 5'-GTGTTGATCCAGCT AGCGGGCGCGATGCGGTGAAG-3' (SEQ ID NO: 38)

YB 5'-TTCACCGCATCGCGCCCGCT AGCTGGATCAACACC -3' (SEQ ID NO: 39)

Mutagenic PCR was conducted using CLONTECH's Genome Advantage 5X Buffer, 1.0 M GCMelt, 1.1 mM MgOAc, 0.2 mM each dNTP, 15 ng of template DNA, and 2.5 units of *Pfu Turbo* DNA polymerase (Stratagene,) in a 50 µl reaction. Plasmid DNA of the X1X2 /pUC19 construct, described above, was used as template. PCR was

conducted according to the manufacturer's specification in the QuikChange™ Site-Directed Mutagenesis Kit, using a 14 minute extension time and 18 cycles of PCR. *Dpn* I treatment and transformation were conducted as per manufacturer's specifications except that 2 μl of *Dpn* I-treated DNA was used in each transformation and cells were recovered in SOC media for 0.5 hour. Cells were plated on LBA plates and plasmid DNA was isolated from ten single colonies of each mutant type. Plasmid DNA of each colony was digested with *Nhe* I restriction enzyme to check for the introduction of a *Nhe I* site introduced through the mutagenic primer. All but one colony had a single *Nhe I* site, compared to the lack of a site in the X1X2Y/pUC19 template plasmid. The presence of the desired mutations and lack of unwanted mutations in other ORFs (i.e., an unwanted mutation in the Y ORF in the X1 mutation vector), were confirmed by sequencing. Plasmid DNA from two mutant colonies for the X1 mutation and one mutant colony for the X2 and Y mutations were used, along with the *idi-crtI*/pPROLarNde vector, in double transformations of electrocompetent cells of *E. coli* strain DH5αPRO. Control transformations using the unmutated X1X2Y/pUC19 vector and the *idi-crtI*/pPROLarNde vector were also conducted. All transformations used 25 ng of the pUC19-based vector and 100 ng of the pPROLarNde-based vector. Cells were recovered for one hour in SOC media and plated on LBAKIA media. Colonies from all of the transformations involving mutant plasmids were red, whereas the control double transformants were yellow. Visible spectral analysis revealed that all the mutant clones (red) produced the C40 carotenoid lycopene while the control double transformant and *A. mediolanus* (yellow) produced the C50 carotenoid decaprenoxanthin (FIG 8).

Hence it was concluded that none of the fragments with mutations in ORFX1, ORFX2 or ORFY, combined with *idi-crtI* fragment were capable of producing a C50 carotenoid.

The results of the three strategies combined with the results from the tests of the previous three constructs (*idi-crtI*, *idi*-ORFX2, and *idi*-ORFY) indicate a significant finding—that the activities of all three ORFs can be used to convert a C40 carotenoid to a C50 carotenoid. If the genes of all three separate ORFs were not present, the conversion of the C40 carotenoid to a C>40 carotenoid was found to not occur.

3. The naming of the ORF genes which allow for the conversion of a C40 carotenoid to a C50 carotenoid

Because the ORFX1, ORFX2, and ORFY genes were all required for the conversion of the C40 lycopene (an acyclic carotenoid) to the C50 decaprenoxanthin (a carotenoid having two ε-ionone rings), the genes have been designated as lycopene ε-cyclase transferases, as described in the following table:

* ORFX1 is designated lycopene ε-cyclase transferase A, or *lctA*.
* ORFX2 is designated lycopene ε-cyclase transferase B, or *lctB*.
* ORFY is designated lycopene ε-cyclase transferase C, or *lctC*.

Based on the data described herein, a biosynthetic pathway for decaprenoxanthin in *A. mediolanus* is shown in FIG 10. It is believed that the genes described herein could be present in other C50 producing bacteria such as *Sarcina flava*, *Corynebacterium poinsettiae*, *Arthrobacter* sp., such as *A. glacialis*, *Sarcina luteus* (*Micrococcus luteus*), *Halobacterium cutirubram* and *salinarium*, and *Cellulomonas biazotea*. It is believed that such genes could be isolated using techniques similar to those used for the present invention, and accordingly, such genes are considered part of the present invention.

IV. Experimental Materials, Methods, Results, and Examples—*Micrococcus luteus*

Brief outline of the subject matter described in section IV

1. Selection of five C50 carotenoid producing bacteria as candidates for study; isolation of genomic DNA.
2. Synthesis of *A. mediolanus lctC* probe from previously described colony Y1.
3. Determination of homology between genes from each candidate bacterium and the lctC probe of *A. mediolanus*.
4. Selection of *M. luteus* ATCC 383 for study in view a substantial homology finding of one of its genes with the lctC probe.
5. Construction of a genomic DNA library for *M. luteus* ATCC 383.

6. Finding substantial homology between lctA, *lctB*, and *lctC* of *M. luteus* ATCC 383 and *lctA*, *lctB*, and *lctC* of *A. mediolanus*.

7. Identification of the carotenogenic operon for *M. luteus* ATCC 383.

8. Sequencing and sequence analysis for the carotenogenic operon.

9. Identification of six genes (*crtE*, *crtB*, *crtI*, *lctA*, lctB, and *lctC*) within the operon.

10. C50 production in *M. luteus* ATCC 383

11. BLAST analyses; Determining homology between genes.

Details elaborating the brief outline are described in the remainder of section IV.

A. Preparation of genomic DNA for candidate bacteria; Choice of *Micrococcus luteus* (ATCC 383)

Five bacteria (species and strains) that produce C50 carotenoids were obtained from ATCC:

* *Micrococcus luteus* ATCC 147.
* *Micrococcus luteus* ATCC 383.
* *Cellulomonas biazotea* ATCC 486.
* *Halobacterium salinarium* ATCC 33170.
* *Halobacterium salinarium* NRC-1.

In addition, the following control was employed

* *Agromyces mediolanus ATCC* 13930 (control).

Genomic DNA was isolated from each line plus the *A. mediolanus* control, using a Gentra Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). Genomic DNA (1.0-1.5 μg) was used in digests with the restriction enzymes *Pst* I and *Xho* I, and separated on a 0.8% Tris-Acetate-EDTA (TAE) agarose gel. DIG-labeled molecular weight markers II and III (Roche Biomedical Products, Indianapolis, IN) were also included on the gel/membrane. DNA was transferred to a nylon membrane using a routine Southern transfer procedure.

DIG-labeled probes (894 bp) of the *A. mediolanus lctC* locus were synthesized using a PCR DIG Probe Synthesis Kit (Roche). Half-strength and full-strength DIG probes were amplified using plasmid DNA of the previously described Y1 clone as

template and the ORFYF and ORFYR primers in 50 μL PCR reactions. The 5' end of the ORFYF primer is located 14 bp upstream of the *lctC* translational start codon and the 5' end of the ORFYR primer is located 15 bp upstream of the *lctC* translational stop codon.

ORFYF: 5'- AGAGGAGCCGAGCGATGAG -3' (SEQ ID NO: 40)
ORFYR: 5'- CGTACCAGATCAGCAGCATC -3' (SEQ ID NO: 41)

The PCR reactions were separated on a 1% TAE-agarose gel and the probes were gel purified using a QIAquick Gel Purification Kit (Qiagen, Valencia, CA). After baking, membranes were prehybridized in EasyHyb Buffer (Roche) for at least 2 hours at 42°C and hybridized overnight at 42°C using 400 nL of the half-strength DIG labeling reaction per mLof hybridization solution. Washing of the membranes and detection of hybridization was achieved using a Wash and Block Buffer Set (Roche). Membranes were washed two times for 5-10 minutes each at room temperature in 2X SSC/0.1% SDS and two times for 15-20 minutes each at 55°C in 0.1X SSC/0.1% SDS. After rinsing with washing buffer, the membranes were covered with blocking buffer and placed on a shaker for 1.5 hours at room temperature. The blocking buffer was replaced with fresh blocking buffer containing 150 mU of AP conjugate per mL of buffer and shaken at room temperature for an additional 30 minutes. Membranes were then washed twice for 15 minutes each at room temperature with washing buffer, followed by a five minute wash with detection buffer. The detection buffer was replaced with fresh detection buffer containing 20 μL of NBT/BCIP solution per mL of buffer. This was placed in the dark at room temperature with no shaking until color developed, after which the buffer was replaced with 10 mM Tris-1 mM EDTA solution.

Of the five strains tested, *M. luteus* ATCC 383 and *M. luteus* ATCC 147 showed fragments having the highest homology to the *lctC* probe. Restriction digests were done of genomic DNA of these two genotypes and *A. mediolanus* using the enzymes *Xho* I, *ApaL* I, and *Sac* I. DNA was separated on a 0.8% TAE-agarose gel, transferred to nylon membrane, and hybridized with the *lctC* probe as described above with the following exceptions. DIG-labeled Marker VII was included on gels/membranes. The DIG-labeled probe, which had been stored at –20°C, was heated at 65°C for 15 minutes before reuse.

After two washes in 2X SSC/0.1% SDS, membranes were washed twice at 64°C in 0.5X SSC/0.1% SDS.

Whereas *M. luteus* ATCC 147 exhibited multiple bands of hybridization, *M. luteus* ATCC 383 showed a single dominant band for most of the digests. The *Sac* I digest for *M. luteus* exhibited a relatively strong band of approximately 4 Kb. Multiple *Sac* I digests were done for this genotype and separated on a 0.8% TAE-agarose gel. DNA fragments approximately 3.5-4.5 Kb in size were excised and gel purified using a QIAquick Gel Purification Kit.

In view of the above findings, *M. luteus* ATCC 383 was chosen for further study.

B. Library construction for *M. luteus* 383; Identification of the carotenogenic operon

The pUC18 vector (2.5 μg) was digested for 3 hours using *Sac* I restriction enzyme to generate fragment ends compatible with the digested genomic DNA from *M. luteus* ATCC 383. The *Sac* I-digested pUC18 was dephosphorylated using shrimp alkaline phosphatase (SAP, Roche Diagnostics GmbH) and subsequently purified using gel electrophoresis on a 0.8% TAE-agarose gel and a QIAquick Gel Purification kit as per the manufacturer's instructions.

Purified insert DNA (60 ng) was ligated with 40-140 ng of prepared vector using T4 DNA ligase at 16°C for 16 hours. A portion of the ligation reaction (1.2 μL) was electroporated into 40 μL of *E. coli* Electromax™ DH10B™ cells using standard electroporation protocols. Transformations were plated on LB media containing 40 μg/mL of X-gal and 100 μg/mL of carbenicillin (LBCX). Once an appropriate plating volume was determined, multiple transformations were conducted using remaining portions of the ligation reaction and were plated to achieve individual colonies.

Individual, white colonies were patched in a 6 x 7 grid to 14 plates of LB with 100 μg/mL of carbenicillin (LBC). Upon growth, colonies were replica plated to new LBC media. Colony lifts were made, according to standard procedures, using one of the sets of plates. Plasmid DNA of the *A. mediolanus* Y1 colony (5 ng) was spotted to some of the membranes as a hybridization control. After baking, each membrane was treated with 600 μL of 1.67 mg/mL Proteinase K (Qiagen) diluted in 2X SSC and heated at 37°C for

1.25 hours. Membranes were then rinsed in 2X SSC on a shaker for one hour at room temperature. Prehybridization, hybridization with the *lctC* probe, membrane washing, and detection of hybridization were conducted as previously described.

Twelve colonies were identified that hybridized above the background level. Plasmid DNA was isolated from cultures of these colonies and digested with the restriction enzyme *Sac* I to check insert size. Six colonies exhibited a single insert and six showed multiple inserts. Four colonies with unique restriction patterns were sequenced using M13R and M13F universal sequencing primers homologous to the pUC19 vector. The M13F sequence of Clone 1, which had a single insert of approximately 3.9 Kb, showed homology to known phytoene desaturases. The remainder of this clone was sequenced by primer walking.

Homologies found for genes of interest are described in more detail in the BLAST Analyses section below. The three ORFs that showed homology to the *lctA, lctB*, and *lctC* genes of *mediolanus* were called *lctA, lctB*, and *lctC* genes of *M. luteus* ATCC 383.

Genome walking was conducted to obtain the sequence of the C50-carotenoid operon upstream of the phytoene desaturase fragment. Genome walk libraries were made according to the protocol described for CLONTech's Universal Genome Walking Kit (CLONTech Laboratories, Inc., Palo Alto, CA). The restriction enzymes *Hinc* II, *Stu* I and *Pvu* II were used in making these libraries. The following primers were used in the procedure:

GSP1F: 5'- TTCATGGACGTGCCCAGCAGCGTTGCCA -3' (SEQ ID NO: 42)
GSP2F: 5'- AGGTGGGCGAAGTCCGTGTAGAGGAAG -3' (SEQ ID NO: 43)

GSP1F and GSP2F are primers facing upstream and GSP2F is nested inside of GSP1F. The addition of 5% DMSO to the PCR mixture was found to be necessary for amplification. First round PCR was conducted in a Perkin Elmer 9700 Thermocycler with 7 cycles consisting of 2 sec at 94°C and 3 min at 72°C and 34 cycles consisting of 2 sec at 94°C, and 3 min at 66°C, with a final extension at 66°C for 4 min. Second round PCR used 5 cycles consisting of 2 sec at 94°C and 3 min at 72°C and 24 cycles consisting of 2 sec at 94°C and 3 min at 66°C, with a final extension at 66°C for 4 min. Nine μL of

the first round product and seven μL of the second round product were run on a 1.5% TAE-agarose gel. A 0.9 Kb band was obtained for the second round product for the *Hinc* II library. This fragment was gel purified using a QIAquick Gel Purification Kit. Four μL of the purified DNA was ligated into pCR®II-TOPO vector and transformed by a heat-shock method into TOP10 *E. coli* cells using a TOPO cloning procedure (Invitrogen, Carlsbad, CA). Transformations were plated on LB media containing 100 μg/mL of ampicillin and 50 μg/mL of X-gal.

Individual, white colonies were screened by PCR using the GSP2F and AP2 primers. Individual colonies were resuspended in approximately 27 μl of 10 mM Tris and 2 μL of the resuspension was plated on LBK media (50 μg/mL kanamycin). The remnant resuspension was heated for 10 minutes at 95°C to lyse the bacterial cells, and 2 μL of the heated cells used in a 25 μL PCR reaction. The PCR mix contained the following: 1X *Taq* buffer, 0.2 μM each primer, 0.2 mM each dNTP, 5% DMSO (v/v), and 1 unit of *Taq* polymerase per reaction. The PCR reaction was performed in a Perkin Elmer 9700 Thermocycler using the same program as used in the second round of genome walking. PCR product was separated on a 1% TAE-agarose gel along with remnant second round *Hinc* II product. Plasmid DNA for two colonies having inserts of the desired size was sequenced with the AP2 and GSP2F primers. The sequence obtained showed homology to known phytoene desaturases.

A second round of genome walking was conducted to obtain the remainder of the C50-carotenoid producing operon. The following primers were designed from the forward end of the sequence obtained from the first round of genome walking:

GSP1F2: 5'- AAGTAGGTGCGTCCGAGCTGGTCGTGGT -3' (SEQ ID NO: 44)
GSP2F2: 5'- GTCCGCGCCGAGATCCCGCAGGAAGTT -3' (SEQ ID NO: 45)

GSP1F2 and GSP2F2 are primers facing upstream and GSP2F2 is nested inside of GSP1F2.

These primers were used in PCR as described above and in the Genome Walker manual. A band of approximately 2.6 Kb was obtained for the second round PCR reaction using the *Pvu* II library. This DNA was gel purified, ligated into pCR®II-TOPO

vector, and transformed into TOP10 *E. coli* cells using a TOPO cloning procedure. Individual colonies were screened by PCR for insert size, as previously described, using the AP2 and GSP2F2 primers. Plasmid DNA was obtained for a colony exhibiting an insert of the desired size and was sequenced using the GSP2F2 and AP2 primers. The remaining sequence for the insert was obtained by primer walking. PCR products for several regions of the operon were also sequenced to confirm the DNA sequence.

The full sequence of the operon, obtained by colony hybridization and genome walking, is given in FIG 12.

As seen in FIG 12, the operon isolated from M. luteus ATCC 383 comprises the following genes in order of location in the operon:

* *crtE*, geranylgeranyl pyrophosphate synthase.
* *crtB*, phytoene synthase.
* *crtI*, phytoene dehydrogenase (phytoene desaturase).
* *lctA* of M. luteus ATCC 383—having homology with *lctA* of *A. mediolanus*.
* *lctB* of M. luteus ATCC 383—having homology with *lctB* of *A. mediolanus*.
* *lctC* of M. luteus ATCC 383—having homology with *lctC* of *A. mediolanus*.

C. Confirmation of C50 production in *M. luteus* ATCC 383

C50 carotenoid (decaprenoxanthin) was produced in *E. coli* when the crtE-lctC gene fragment from *M. luteus* was cloned into *E.coli* together with the *idi* gene from *E.coli* on a pUC19 plasmid.

A gene construct containing the *crtE, crtB, CrtI, lctA, lctB* and *lctC* genes were inserted into the expression vector pProLarNde as described above. The *idi* gene from *E. coli* was cloned into the vector pUC19. These two plasmids were co-transformed into *E.coli* DH10B electrocompenet cells. Approximately 60 ng of the idi+pUC19 construct and 240 ng of crtE-lctC+pPRONde construct were used to electroporate 40 µL of ElectroMAX DH10BTM competent cells. Electroporated cells were recovered in SOC

media for one hour and plated on LB plates containing 50 μg/ml of kanamycin, and 50 μg/ml of carbenicillin. Colonies were obtained after incubation at 37°C and plated on LB plates containing 50 μg/ml of kanamycin, and 50 μg/ml of carbenicillin,1 mM IPTG, and 2% L-arabinose (LBKCIA) to induce gene expression from both vectors. After incubation colonies were scraped off the plate and extracted by the DMSO method of An et al. Cells were washed once with distilled water and once with acetone. The pellets were dried in air and resuspended in one ml of DMSO preheated to 55°C. Glass beads were added to each tube and vortexed to resuspend the pellets. One ml of acetone was added to extract the carotenoid , and one ml of hexane and two mls of 20 % sodium chloride solution were added and the tubes vortexed. The phases were separated by centrifugation and the hexane phase was removed for carotenoid analysis. Spectrophotometric analysis between 350 and 500 nm revealed that the carotenoid profile matched that expected for decaprenoxanthin. These hexane carotenoid extracts were also subjected to mass spectrometer analysis and the expected Mass ion of 705.3 was observed in the *E.coli* double transformant as well as two additional mass ions at 687.4 and 669.6 corresponding the loss of one and two water molecules respectively. This mass of 705 (M+H) matches that expected for decaprenoxanthin.

D. BLAST analyses to determine homology between genes

BLAST searches of the above DNA sequence for *M. luteus* ATCC 383 against the Swisspro database identified the probable translational start and stop codons for the genes in the C50-carotenoid operon. The geranylgeranyl pyrophosphate (GGPP) synthase gene (*crtE*) for *M. luteus* ATCC 383 showed highest homology to the GGPP synthase gene of *Brevibacterium linens* (33% identity). The *M. luteus* ATCC 383 phytoene synthase gene (*crtB*) had highest homology to the phytoene synthase gene of *Corynebacterium glutamicum* (31% identity), followed by that of *Brevibacterium linens*. The phytoene desaturase gene (*crtI*) of *M. luteus* ATCC 383 showed highest homology to phytoene desaturase/dehydrogenase genes in *Brevibacterium linens, Corynebacterium glutamicum, Halobacterium salinarium* NRC-1, and *Methanobacter thermautotrophicus*, in order of decreasing homology.

The only significant BLAST hit for the *M. luteus* ATCC 383 *lctA* and *lctB* genes were to epsilon cyclase genes in *Corynebacterium glutamicum* (*crtYe* and *crtYf*, respectively, of Krubasik et al., *Eur. J. Biochem.* 268: 3702-3708 (2001)). The *lctC* gene of *M. luteus* ATCC 383 showed homology to lycopene elongase (*crtEb* of Krubasik et al.) from *Corynebacterium glutamicum*, followed by ORFs in *Deinococcus radiodurans* and *Halobacterium salinarium* NRC-1.

Alignments of genes from *M. luteus*, *A. mediolanus*, and *C. glutamicum*)

Alignments for the *crtE* (GGPP synthesis genes), *crtB* (phytoene synthase genes), *crtI* (phytoene desaturase gene), *lctA, crtYe, lctB, crtYf, lctC*, and *crtEb* genes from *M. luteus* (Ml), *A. mediolanus* (Am), and *C. glutamicum* (Cg) were aligned. Alignments were done using Align Plus software (Scientific and Educational Software, Durham, NC). These alignments were done using the multiway protein alignment function in conjunction with the BLOSUM 62 matrix.

Results indicate that there is significant sequence identity shared between the amino acid sequences. These results indicate that the sequences could be used as substitutes for each other when they are used to create biosynthetic routes for generating C40, C45, and/or C50 carotenoids. Tables 3-8 provide a summary of the results from the alignments.

Table 3

Gene	Start	End	Length	Matches	% Sequence Identity
M1-*crtE*	1	366	366 aa	188	49% (M1-*crtE* and Am-*crtE*)
Am-*crtE*	1	369	369 aa	207	54% (Am-*crtE* and Cg-*crtE*)
Cg-*crtE*	1	382	382 aa	158	40% (Cg-*crtE* and MI-*crtE*)

Table 4

Gene	Start	End	Length	Matches	% Sequence Identity
Mi-*crtB*	1	331	331 aa	190	56% (MI-*crtB* and Am-*crtB*)
Am-*crtB*	1	303	303 aa	178	56% (Am-*crtB* and Cg-*crtB*)
Cg-*crtB*	1	304	304 aa	304	47% (Cg-*crtB* and MI-*crtB*)

Table 5

Gene	Start	End	Length	Matches	% Sequence Identity
Mi-*crtI*	1	543	543 aa	337	59% (MI-*crtI* and Am-*crtI*)
Am-*crtI*	1	544	544 aa	364	65% (Am-*crtI* and Cg-*crtI*)
Cg-*crtI*	1	549	549 aa	308	54% (Cg-*crtI* and MI-*crtI*)

Table 6

Gene	Start	End	Length	Matches	% Sequence Identity
Mi-*lctA*	1	115	115 aa	62	52% (MI-*lctA* and Am-*lctA*)
Am-*lctA*	1	123	123 aa	67	45% (Am-*lctA* and Cg-*crtYe*)
Cg-*crtYe*	1	132	132 aa	62	48% (Cg-*crtYe* and MI-*lctA*)

Table 7

Gene	Start	End	Length	Matches	% Sequence Identity
Mi-*lctB*	1	164	164 aa	69	44% (MI-*lctB* and Am-*lctB*)
Am-*lctB*	1	115	115 aa	66	36% (Am-*lctB* and Cg-*crtYf*)
Cg-*crtYf*	1	130	130 aa	53	42% (Cg-*crtYf* and MI-*lctB*)

Table 8

Gene	Start	End	Length	Matches	% Sequence Identity
Mi-*lctC*	1	291	291 aa	206	66% (MI-*lctC* and Am-*lctC*)
Am-*lctC*	1	298	298 aa	199	57% (Am-*lctC* and Cg-*crtEb*)
Cg-*crtEb*	1	287	287 aa	166	70% (Cg-*crtEb* and MI-*lctC*)

V. Conclusions

The experiments described above allowed for the isolation of the following seven (7) genes involved in the biosynthesis of the C50 carotenoid decaprenoxanthin in *A. mediolanus*:

* isopentenyl pyrophosphate (diphosphate) isomerase (*idi*),
* geranylgeranyl pyrophosphate synthase (*crtE*),
* phytoene synthase (*crtB*),
* phytoene desaturase (*crtI*),
* lycopene ε-cyclase transferase A (*lctA*),
* lycopene ε-cyclase transferase B (*lctB*), and
* lycopene ε-cyclase transferase C (*lctC*).

Similar genes with substantial homology to the *A. mediolanus* genes were then isolated from *M. luteus*. It is believed that other similar genes with substantial homology could be isolated using similar techniques, and that such genes fall within the present invention.

The experiments also show that there is a conservation in the gene arrangement between ORFs X1, X2 and Y, or *lct A, B* and *C* genes respectively. A schematic comparison of the *lct A, B* and *C* genes from *A. mediolanus* and *M. luteus* with certain genes from other bacteria is shown in FIG 9.

A schematic biosynthetic pathway, which is believed to summarize reactions of the present invention, is shown in FIG 10. As has been shown, the *lct* genes code for enzymes that react with the C40 carotenoid lycopene to perform two successive ε-cyclizations—coupled to the addition of C5 residues at the 2 and 2' positions of the resulting carotenoid—to form (successively) a C45 (dehydrogenans-P452) and a C50 (decaprenoxanthin) carotenoid.

The invention provides genes capable of converting a C40 carotenoid to a C50 carotenoid. These genes (*lctA, lctB,* and *lctC*) are the first example of a set of genes that covert at C40 carotenoid to a C50 carotenoid in a single step. The three separate proteins can be used to convert a C40 carotenoid to the C50 carotenoid in a single step.

Some alternate uses of the genes described in this report are listed below. Some or all of the identified genes involved in lycopene biosynthesis (*crtE, crtB, crtI*) could be used alone, or in combination with carotenogenic genes from other organisms, in order to produce carotenoids such as (but not limited to): lycopene, ß-carotene, lutein, zeaxanthin, canthaxanthin or astaxanthin. The gene for isopentenyl pyrophosphate isomerase (*idi*) could be utilized to increase the concentration of any carotenoids produced by a microorganism. This *idi* gene could be used in a genetic background that includes none, some or all of the other *A. mediolanus* carotenoid biosynthetic genes described here. A gene for carotenoid glycosyl transferase (e.g., zeaxanthin glycosyl transferase (*crtX*)) in a genetic background capable of producing dehydrogenans P-452, may be used to produce dehydrogenans P-452 monoglucoside; or (in a decaprenoxanthin producing background) to produce corynexanthin (decaprenoxanthin monoglucoside) or corynexanthin monoglucoside. Use of a carotenoid desaturase gene that is capable of adding additional conjugated double bonds to the C50 substrate will increase the antioxidant capacity of the molecule and change the spectral properties of the molecule (i.e. increasing the $_{max}$ of the carotenoid). As mentioned before, sequence similarity searches of the Genbank public databases show three genes which have certain levels of homology to *lctC*. These genes are from carotenogenic organisms (*Deinococcus radiodurans, Halobacterium sp.* NRC-1, and *Methanobacterium thermoautotrophicum*) but their functions had not been previously defined. Because of the level of similarity between the gene sequences, it is probable that these three genes define a family of genes, all of which are involved in the conversion of C40 carotenoids to C>40 carotenoids. The *lct* genes may be manipulated to perform other, related functions. These may include (but are not limited to): addition of the C5 residue without the associated cyclization reaction and/or addition of the C5 residue with a β-cyclization reaction (as opposed to the current ε-cyclization).

It is not difficult—through the use of additional enzymes like the FGPP synthase, combined with the genes isolated from *A. mediolanus*—to generate a fully conjugated

novel C50 carotenoid with greatly improved antioxidant potential as well as unique absorption maxima. Such a molecule would result in carotenoids with novel colors. Similarly, modified phytoene desaturases—created by shuffling or by using other mutagenic techniques—could be employed with concepts of the present invention to create additional high performance carotenoids.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated polypeptide comprising at least one amino acid sequence selected from the group consisting of:

(a) the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26;

(b) an amino acid sequence having at least 10 contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26;

(c) an amino acid sequence having one or more conservative amino acid substitutions within the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; and

(d) an amino acid sequence having at least 65% sequence identity with the amino acid sequences of (a) or (b).

2. An isolated nucleic acid molecule encoding said polypeptide of claim 1.

3. The nucleic acid molecule of claim 2, wherein said polypeptide is capable of converting a C40 carotenoid to a C50 carotenoid.

4. The nucleic acid molecule of claim 2, wherein said polypeptide is capable of converting a C40 carotenoid to a C45 carotenoid.

5. The nucleic acid molecule of claim 2, wherein said polypeptide is capable of converting a C45 carotenoid to a C50 carotenoid.

6. The polypeptide of claim 1, wherein said polypeptide is capable of synthesizing a C40 carotenoid.

7. A production cell comprising said nucleic acid molecule of claim 2.

8. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23;

(b) a nucleic acid sequence having at least 10 contiguous nucleotides of the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23;

(c) a nucleic acid sequence that hybridizes under moderately stringent conditions to the nucleotide sequence of (a); and

(d) a nucleic acid sequence having 65% sequence identity with the nucleic acid sequence of (a) or (b).

9. A production cell comprising said nucleic acid molecule of claim 8.

10. A method for making a C50 carotenoid, said method comprising contacting at least one of said polypeptides of claim 1 with a C40 carotenoid such that said C50 carotenoid is made.

11. A method for making a C50 carotenoid, said method comprising culturing said production cell of claim 7 under conditions wherein said C50 carotenoid is made.

12. A method for making a C45 carotenoid, said method comprising contacting at least one said polypeptide of claim 1 with a C40 carotenoid such that said C45 carotenoid is made.

13. A method for making a C45 carotenoid, said method comprising culturing the production cell of claim 7 under conditions wherein said C45 carotenoid is made.

14. A method for making a polypeptide, said method comprising culturing said production cell of claim 7 under conditions such that said polypeptide is made.

15. A specific binding agent that binds to said polypeptide of claim 1.

16. A method for making a C>40 carotenoid, said method comprising culturing a production cell, wherein said production cell comprises an exogenous nucleic acid molecule, wherein said exogenous nucleic acid molecule encodes a polypeptide that elongates a C>40 carotenoid by at least one carbon atom, wherein the product produced by said polypeptide is a carotenoid having a carbon backbone of >40 carbon atoms.

17. The method of claim 16, wherein said exogenous nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23;

(b) a nucleotide sequence having at least 10 consecutive nucleotides of the nucleotide sequence set forth in SEQ ID NOS: 01, 02, 03, 07, 08, 09, 13, 14, 15, 16, 21, 22 or 23;

(c) a nucleic acid sequence that hybridizes under moderately stringent conditions to the nucleotide sequence of (a); and

(d) a nucleic acid sequence having 65% sequence identity with the nucleic acid sequence of (a) or (b).

18. The method of claim 16, wherein said exogenous nucleic acid molecule encodes a polypeptide, said polypeptide comprising at least one amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26;

(b) an amino acid sequence having at least 10 contiguous amino acid residues of the amino acid sequence set forth in SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26;

(c) an amino acid sequence having one or more conservative amino acid substitutions within the amino acid sequence of SEQ ID NOS: 04, 05, 06, 10, 11, 12, 17, 18, 19, 20, 24, 25 or 26; and

(d) an amino acid sequence having at least 65% sequence identity with the amino acid sequences of (a) or (b).

Agromyces mediolanus carotenogenic operon

Proposed translational start codons (bolded)

idi	1888
crtE	2505
crtB	3611
crtI	4584
ORFX1	6215
ORFX2	6583
ORFY	6927

Proposed translational stop codons (underlined)

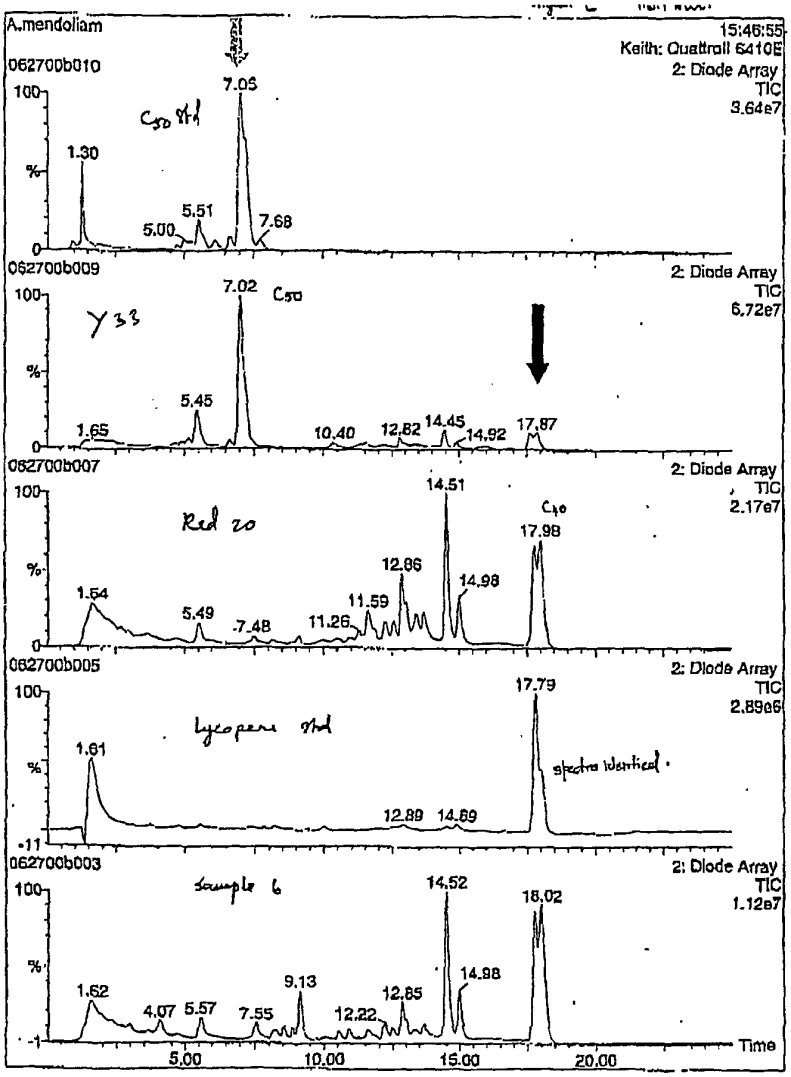
idi	2508
crtE	3614
crtB	4587
crtI	6218
ORFX1	6586
ORFX2	6930
ORFY	7823

```
   1  ggatcacggg cagctcgacg ccgcgccggg cgagctcggc ctcgagtgcg gccttcagct
  61  cgcggttctg ctggttgatc gggctgatgc cgtcgaagtg gcggtagtgg tgggcgacct
 121  cttcgaggcg ctcgtcgggg atgccccggc cgctcgtgac gttccgcagg aaggggatga
 181  cgtcgtcctg ccgctcgggc ccgccgaagc cggccagcag gatcgcgtcg taggcgacgg
 241  gctcggtgac gtgctcgggg cccgactggg cggcctcggt ggcaccgggc acgcaggcgc
 301  ccgaggcgca gtacgcctcg gcggcggggg ccggcttgcg gccgcgggcg gcctcgcgct
 361  cgggcgcggc ggctccggtc gagcccaggt tcgtcgcggc cattactgga gcaccrccac
 421  gagctcggcg gtcgagatcc gtcgaccggt gtagaacggg acctcttcgc gcacgtgcat
 481  gcgggcgtcg gtggcgcgca gctcgcgcat gaggtcgacg agctcggtga gctcgtcgga
 541  ctcgatgggc agcagccact cgtagtcgcc gagggcgaag gcgctcacgg tgtgggcgat
 601  cgcgccgcgg aaggtggcgc ccttgcggcc gtggtcggcg agcatgcggg agcgctcggc
 661  cgggtcgagc aggtaccagt cgtagctgcg cacgaagggg tagaccgtca gccagccctt
 721  gggctcgatg ccgcgcagga agccccggcac gtgcgccttg ttgaactcgg cgtcgcggtg
 781  cacgcccatg gcgttccacg tcggcagcag cgcgcgcagc aggcggctgc gcttcagctc
 841  gcgcagcgcc cactgcaggc cctcggcggt ggcgccgtgc agccagatca tgacgtcggc
 901  gtcggcgcgg aggccggaga cgtcgtagag cccgcgcacc gtgacgccct cgttctcgac
 961  gagcgcgatg acgccgtcga gttcggtcac gaagcgcggc acatcgcgcc cgtcgaggtc
1021  atcggggcgc gcggggtcct ttcggagcac ggcgaagagc gtgtagccct cgggcgactg
1081  ctcgggttcg gacgcgtgac ggagctcgtc ggctgccccc tcggcagcgg gggaagacat
1141  acccccagtc tccctctttc ccccggaagg tccaaaaggg aggcgtcggc tccgccgaat
1201  ggcgcgggaa tccgcggacg gctcagtcct gtccggtcgc ggcgagcgcg tcgaggaagc
1261  ggatgacgac ctcgcgctcc tccggcgcga gggccgccgc ggcggcgaag cgtcgcgcgt
1321  gctgacggcc gacggtctcg cgggcgtcgt gtcgggtgtg ctcggtgacg gcgatcgcga
1381  gggcgcggcg gtcgctcggg tggggcgatc gggtgacgtg gccgccgcgt tcgagccggt
1441  cgaggagctt cgtggtggag gcgctggaga tgccgaggtg ctcggcgagc gcgccgggcg
1501  tcacgacgag cccctggttg cgtgcggcga tgaggaagcg gatggcgcgc atgtcggtct
1561  ggttgagctg catgtagcgc cgggatgcct cgctcatgcg ctcggcggcg gcgtgccagc
1621  cgcgcagcgc ctgcatgacg cgcacgacct ggtcgacctc ctcgtcggcg agtccgctgc
1681  ggtcgacgag ctcctcgtcg cggtcgacga tgcgcggatc gtgcatcgcc gattccacgc
1741  gccggctgcg ctcccgctca tctgccatgt cgagattcta gccaagcgag acgaatctcg
1801  ctaagctact cactagccag gcgagatatt cgccgcagcg agggttcgga tcgagcacct
1861  cgcgccggag ttgtcgaagg agccgacatg accgacctca gcatcacgcc gctgccggcc
1921  caggccgcac cggtgcagcc cgcatccagc gccgaattgg tcgtgctgct cgacgaggcc
1981  ggcaaccaga tcggcaccgc cccgaagtcg agcgtgcacg gcgccgacac cgccctccat
2041  ctcgcgttct cctgccacgt cttcgacgac gacgnccgcc tcctggtgac ccgtcgcgcg
2101  ctcggcaagg tcgcctggcc cggcgtgtgg accaactcct tctgcgggca ccccgccccg
2161  gccgagccgc tgccgcacgc ggtgcgccgc cgggccgagt tcgagctcgg cctcgagctc
2221  cgcgacgtcg agccggtgct gccgttcttc cgctaccggg cgacggatgc ctcgggcatc
2281  gtcgagcacg agatctgccc ggtctacacg gcgcgcacaa gctcggtgcc ggcgccgcat
2341  cccgacgagg tcctcgacct cgcctgggtc gaaccgggcg agctcgccac cgcggtccgc
2401  gccgcgccct gggcgttcag tcgctggctc gtgctgcagg cgcagctgct gcccttcctc
2461  ggcggccacg ccgacgcgcg cgtccgcacg gaagcgctcg tctcgtgagc ctcgtcgcga
2521  ccgtggtcgc cccgagccgg caggcggagg tggagcgcta cctcggcggc ttcttcgacg
2581  acgccatcgt gcgggccgac gcgcacgccg ccgactaccg gcggctctgg gcggcggcgc
2641  gggacgccgc gagcggcggc aagcggatcc gccccaggct cgtgctgggc gcctacgacg
```

```
2701 cgctcgccgc gcagggtgcg ccggcgagcg gccgcgaacg ggccgacgcc gagccggccg
2761 ccgccgcgga ggccgtggcg ctcgcggcgg ccttcgagct gctgcacacc gcgttcctcg
2821 tgcacgacga cgtcatcgac cgcgacctcg tgcgccgggg cgagcccaac gtcgccggcc
2881 gcttcgcgct cgacgccgcg ctgcgcgggc tcgagcggga gcgggcggac gcctacggcc
2941 aggcctcggc gatcctcgcg ggcgacctgc tgatcgcggc ggcgcactcc gtggcggccg
3001 cctcgacgtg ccggtcgagc gccggcgagc catcctcgcc gtccttgacg aagtgcgtct
3061 tcgccgccgc cgcgggcgag cacgccgacg tccggcacgc cgccggggtg cggccccggg
3121 aggcggacat cctcgcgatg atcgaggaca agacggcctg ctactcgttc agcgcgccgc
3181 tccgggcggg cgcgctgctc gccggcgccc cgcgcgcgac ggtcgaacgg ctcggcgaga
3241 tcggccgtcg actcggcgtc gccttccagc tgcaggacga cgtgctcggc gtctacggcg
3301 acgagcgggt gaccggcaag acggcgctcg gggacctccg cgagggcaag gagacgctgc
3361 tcatcgccta cgcgcggggg cacgcggcct gggtcgcggc atccggcgcc ttcggccggc
3421 ccgacctcga cgaggcgggc gcccgccccc tccgcgcggc gatcgaggcg agcggcgccc
3481 gcgcccgcgt cgaggcgcgc atcgccgagg aggcggccgc ggcgcgcacg gcgatcgccg
3541 cggcgggcct gcccgccgcg ctcgaagccg agttgctcgg cctcgccgcc gaagccacca
3601 ggaggtcgag gtgaccgcgc tcccgatcgg cgctgcgttg ctcggcctcg ccgccgaagc
3661 caccaggagg accaggtgag cacgcgcacc acccagcgca cgaccgcgcc gcccgcaccg
3721 tccaccggcc tcgccctcta cgaccgcacc gccgccgagg gctcggcccg ggtcatccgg
3781 gcgtactcga cctccttcgg cctcgcgagc cggctctgct ccccgccgt ccgcgagcac
3841 ctcgccgagg tctacgcgct cgtgcgcatc gccgacgagc tcgtcgacgg cccggccgag
3901 gaggccgggc tgccgtgcga gcgccgccgc gagctgctcg acgccctcga ggccgacacg
3961 gaggccgcct tcgagagcgg ctacagcgcc aacctcgtgg tgcacgcctt cgcgcgcgcg
4021 gcgcggcgca gcggcttcgg ccaggagctc acccggccct tcttcgcctc gatgcgacgc
4081 gacctcgagc ccatcgcctt caccgaggag cgcgagctcg acgaatacgt ctacggctcg
4141 gccgaggtcg tcggcctgat gtgcctgcgc ggcttcgcga tcgggctcgc cccgacgcc
4201 gagcgcgacg cccgctggga gcgcggcgcg cgggcgctgg gctcggcgtt ccagcgggtc
4261 aacttcctgc gggacctcgg ggaggatgcc tcgctccgcg gacgccgcta cttcccgggc
4321 gtcgatccgg tgagcttctc ggaggcccag caactgcgcc tcctcgacgg catcgacgcg
4381 gagctcgacg aggcggccgc cgtgatcccg gagctgcccc gcggctgccg cgtcgcggtc
4441 gccgcggcgc acggcctgtt cggcgagctc tccgccggcc tccgccgcac gcccgcggcc
4501 gagctcgtca cccggcgggt ccgggtgccc gcgccgcgca agctcgccat cgtcacccgc
4561 gtggtcgccc gcggaggccg gccgtgagcc gcgcggtcgt catcggcggc ggcatcgccg
4621 ggctcgccac ggcggcgctg ctcgcccgcg acgggcacga ggtgcggctc ttcgaggcgc
4681 gcgacgagct cggcggccgt gccgggcgct ggcgggcgaa cggcttcctg ttcgacaccg
4741 gtccgagctg gtacctcatg ccagaggtgt tcgagcactt ctaccgcttg atgggcacca
4801 cggcggccga ggagctcgag ctcgtgcgcc tcgaccccgg ctaccgggtg tacttcgagg
4861 gctacgacga gccggtcgac gtgcgggccg agcgcgaggc atccatcgcc ctcttcgagt
4921 cgatcgagcc gggcgcgggc gccgcgctcg cccggcacct cgactccgcc aacgagacgt
4981 accggctcgc gatgacgcac ttcctctaca ccgacttcgc ccaccgggg gcgctgctcg
5041 ccgcgccggt ccggccgcgg ctcggccggc tcgcgaagct gctgctcgaa ccgctcgacc
5101 gcatggtggg gcgctccttc gacgacgtgc ggctgcggca gatcctgggc tacccggcgg
5161 tcttcctcgg cacctcgccc gagcgggcgc cgagcatgta ccacctgatg agccgcttcg
5221 acctcgccga cggggtgttc taccgatgg gcggcttcgg cgagatcatc gcgagcgtgg
5281 cccggctggc ccggcgggcc ggggccgagc tcgtcaccgg cgcgcgggtg ctcggcatcg
5341 agacggccgg cgggcgcgcc acgggcgtgc gcgtgcagca ccacggcccg accggtggca
5401 ccggcaccga ggagttcctg gaggccgagc tcgtcgtctc cgccgccgat ctgcatcaca
5461 cggatgccga gctgctcccg ccccgcgcgc ggacgcggag cgaggcatcc tggtcgcgcc
5521 gcgacccccgg acccggcacg gtgctcgtca tgctcggcgt gcacgggcgg ctgccggagc
5581 tcgcccacca cacgctctgc ttcacggccg actggcgcac gaacttccag cgggtgttcg
5641 gctcgcgacc ggcgatcccc gacccggcgt cgttctacgt ctgccgcccg agtgcgacgg
5701 atccgggcgt ggcgcccccc ggctgcgaga acctgttcct gctcgtgccg gtgcccgccg
5761 acccccacaat cggcgccggc ggtgtcgacg gccgcggcga ccgggcgggtc gaggagacgg
5821 ccgaccgggc gatcgcgacc ctcgccgagt gggccggcat ccccgacctc gccgagcgga
5881 tcctcgtgcg ccgcacgatc gggcccgcgg acttcgagga ctggttccag tcctggcgcg
5941 gctcggcgct cggcccgggg cacacccctgc ggcagagcgc catgttccgg gggcgcacgg
6001 cctcggcgaa cgtcgagggg ctgtacttcg cgggggcgac gacgatcccg ggcatcggcc
6061 tgccgatgtg cctgatcagc gccgagctcg tcgcgaaggc cgtgcgcggc gaggatgccc
6121 cgggcccgct cccggagccg agcgaggagc cgcacccaga cccgctgcac ccagacccgc
6181 tgcacccaga ccggctcgac cgggagcgca ccggatgacc ttcctccacc tggggctgct
6241 gctcgcctcg atcgcgtgca tcgcgctcgt cgacgcgcgc taccggatgt tcttctggcg
6301 ggcgccgctg cgggcgacgg tcgtggtcgc cctcggcgtc gcgatgctcc tcgtctgggg
6361 cctctggggc atctcgctcg gcatcttctt ccgcgagccg aatgcctact cgacgggggct
6421 gctcattgcg ccgcacctgc cgatcgagga gccggtgttc ctcgccttcc tctgccagct
6481 cgcgatggtc ggctacacgg gactgctgcg cctcctcgcg caccgatccg cgcagccccgc
6541 caccggcccc gctgccgact ccaccgccga aggggcccgc cgatgagcta cgccgtgctc
6601 tgcctcccgt tcctcgccgt ctcggcggtg ctcgccgcga tcgcctggcg acgtgctccg
6661 gccggtcacg cggccgcgct cgcgctcacg gcgggcggcc tcgtgctcct caccgcggtg
```

```
6721  ttcgactcgc tgatgatcgc cgcgggcctg ttcgactacg ccgacgcgcc cctgctcggc
6781  ccgcgcctcg ggctcgcccc gatcgaggac ttcgcctacc cgatcgccgc gctgctgctc
6841  tgctccacgg tctggacgct gctcgggcga gcggatgcct cggcggctcg tgaccggccc
6901  gcccgcgcgc ccagaggagc cgagcgatga gcgccgtcgg cgccgaggca tccggccagc
6961  gcctgctccc cgcgctcttc accgcatcgc gcccgctgag ctggatcaac accgccttcc
7021  cgttcgcggc cgcgtacctg ctgaccgtgc gcgaggtcga cgtcgcgctc gtcgtcggca
7081  ccctgttctt cctcgtgccg tacaacctcg cgatgtacgg catcaacgac gtcttcgact
7141  tcgagtccga cgcgcggaat ccgcgcaagg gcggcgtcga gggggccctg ctgccgcccg
7201  cccggcatcg cgcggtgctg atcgccgcgg tggccctgac ggtgccgttc gtcgtctggc
7261  tcgtgctgct cggcggcccg tggtcgtggg cctggctcgc gctcagcctg ttcgccgtgg
7321  tggcgtactc ggcgccgggc ctcaggttca aggagatccc ggggcctgac tccctcacct
7381  cgagcacgca cttcgtctcg cccgcctgct acgggctcgc cctcgcgggg gcgacggtga
7441  cgccgcagct cgtgctgctg ctgctcgcgt tcttcgtgtg gggcgtcgcg agccacgcct
7501  tcggcgcggt gcaggacgtc gtgcccgatc gcgaggccgg gatcgggtcg atcgcgaccg
7561  cgctgggggc ccgccgcacg acccggctcg cgatcggcct ctggctgctc gcgggcgtgc
7621  tgatgctcgg cacgtcgtgg ccggggccgc tcgccgcggt actcgccgtg ccgtacctcg
7681  tcgcggcgtg gccgtaccgc tcggtgagcg acgccgagtc ggcgcgcgcg aacggcggct
7741  ggcgctggtt cctcgcgatc aactacggcg tcggcttcgc ggcgacgatg ctgctgatct
7801  ggtacgcgct gctcacggcc tgagccgtcg ctccgcggng agggcgcgag tccgcgagcg
7861  cgtcactgcc cgtcgagggg cgtcactccc cgtcgagggg cggcgatccg agcaggagcc
7921  cggtcgagtg ggcgatgtgc cgccgcatcg cctcgacgcc ctcggcctgc acctcggcga
7981  gcagctcgcg gtgctcgacg acgagttcgg cgaggctgta gtgccggcgg atgtgcagca
8041  ggaagagccg cagctcggcg ccgagcgcgg cgtgcgcctc gacgatcctg gggctgccgc
8101  tggcggcgac gatcgcccgg tgcacctcga ggtggagccg ctcggcctcg agccaggccg
8161  gggtcgtctc gcgctcgccc ggaggacgca gcgcctcctc ggtgacggcg agccgggcga
8221  gctcgtcgag ggcgagcatc gccggggcga gcgccgcctc gggccagtgc gcgccgtagc
8281  ggtcgcccgc gatgcgtacc gcttcgacct cgagcgcctc gcgcagttgc tgcagcgcga
8341  gcacctgcgc gtggtcgaac tcggtgaccc gcactccgcg gtagggcgcc gactcggcga
8401  gccgctcagc gacgagccgc tggaacgcgg cgcgcacggt gtgccgggac acccgaagc
8461  gctcggccgc ctgctcctcg cgcagcgggg ccccgaggc cagcgcgccg ctcaggatct
8521  cgtcacggag cgcatcggcc atccgctcga cggcggtggg cgccggcatg acgcggcggc
8581  tcagtcgtcg ctgacggcag cgcgcacgac gagggcgacg acgccggcga ccacgacgac
8641  cgccccgatc c
```

Continued **FIG. 1**



A mediolanus

idi-Y clone

idi-crtI clone

Lycopene std

idi-X2 clone

FIG. 2

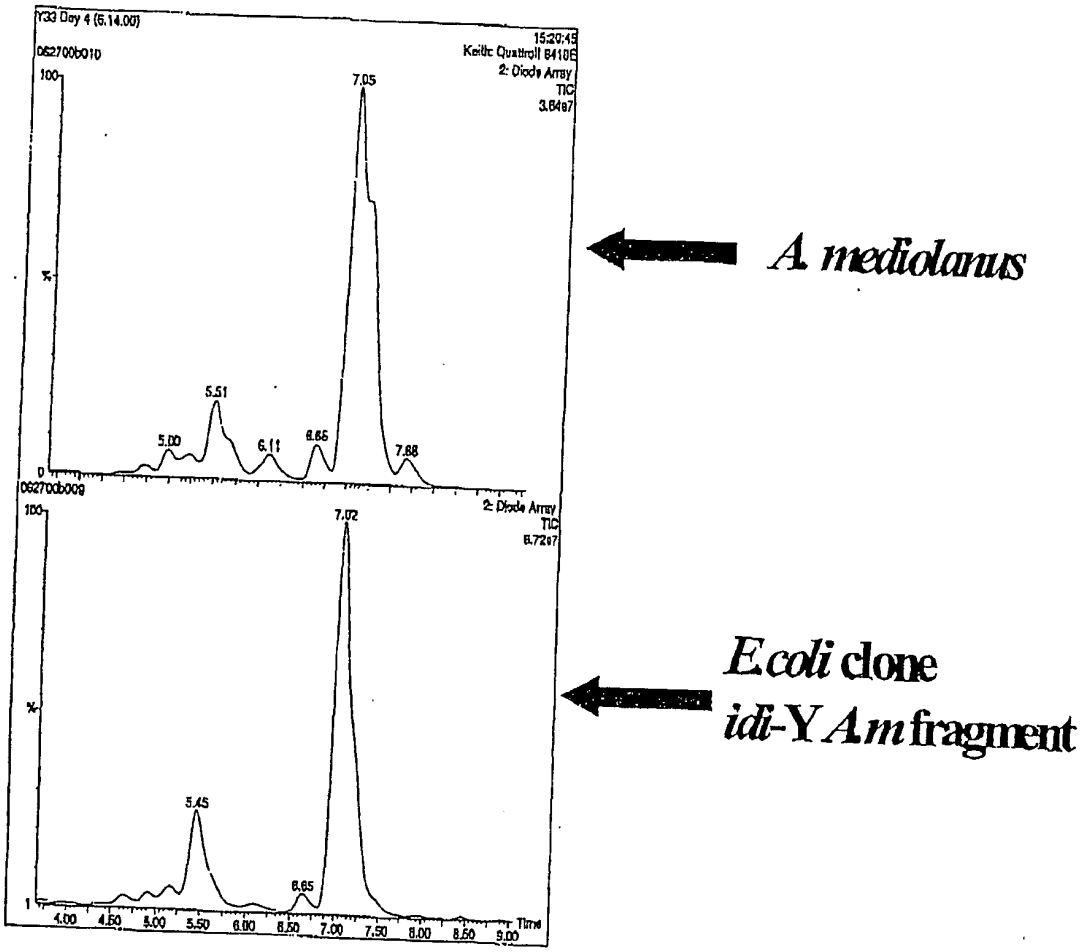
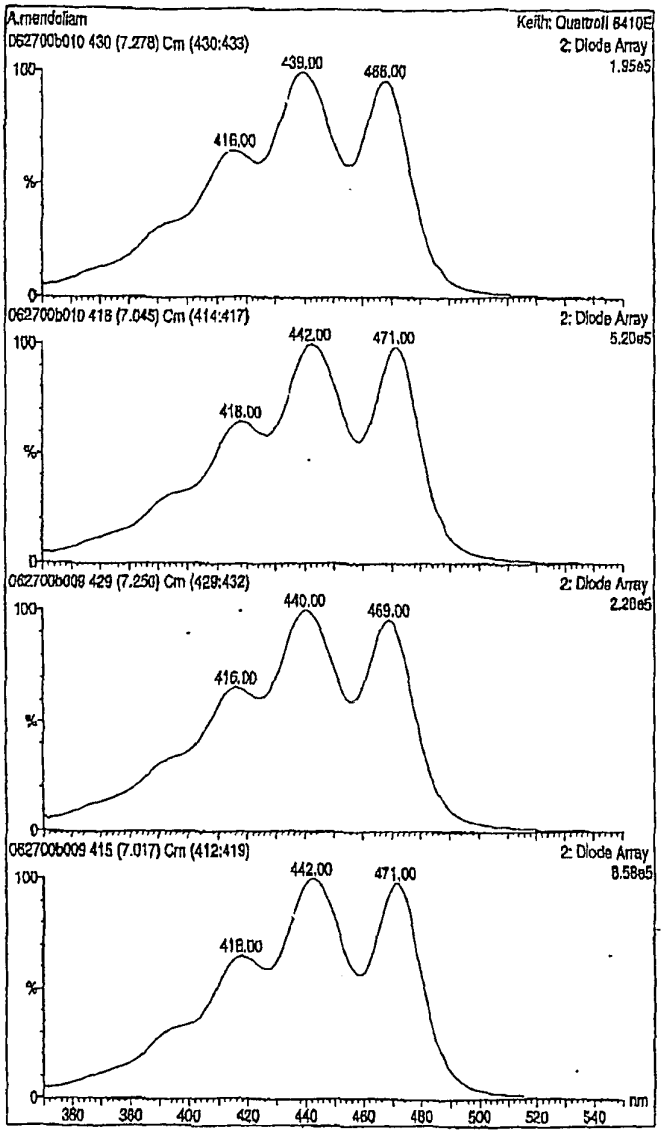

Y33 Day 4 (5.14.00)
062700b010
15:20:45
Keith: Quattro II 8410E
2: Diode Array
TIC
3.6487
100
%
0
7.05
5.51
5.00
6.11
6.65
7.88
062700b009
2: Diode Array
TIC
6.72e7
100
%
1
7.02
5.45
6.65
4.00
4.50
5.00
5.50
6.00
6.50
7.00
7.50
8.00
8.50
9.00
Time
A. mediolanus
E.coli clone
idi-YAm fragment

FIG. 3A



A. mediolanus

E.coli clone
idi-Y*Am* fragment

FIG. 3B

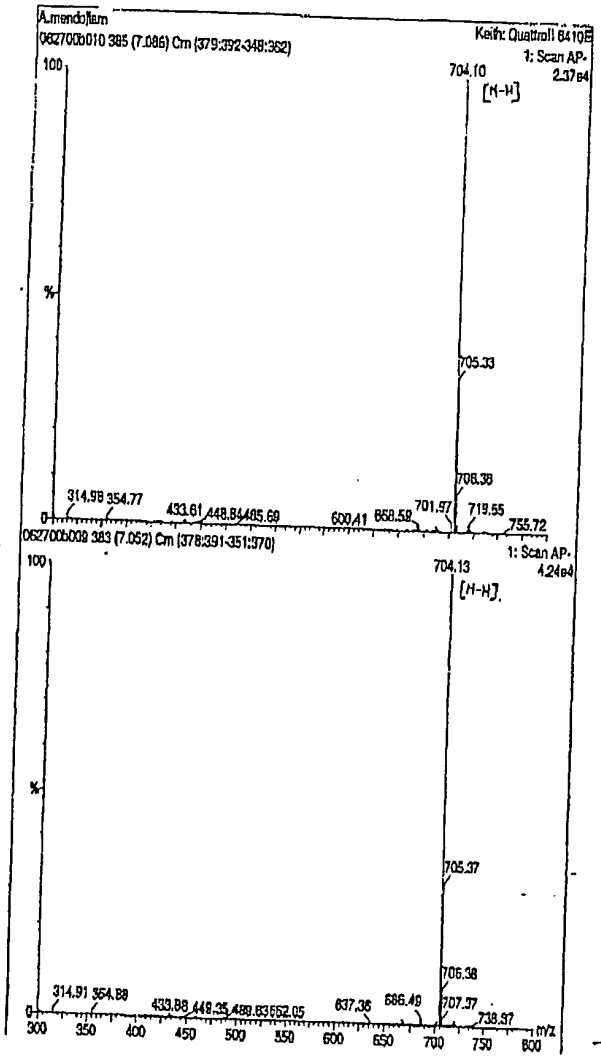

A.mendofilum
062700b010 385 (7.086) Cm (379:392-348:362)
Keith: Quattro II 6410E
1: Scan AP-
2.37e4
100
704.10
[M-H]
%
705.33
706.39
314.98 354.77
433.61 448.84 485.69
600.41 658.58
701.97
719.55
755.72
062700b009 383 (7.052) Cm (378:391-351:370)
1: Scan AP-
4.24e4
100
704.13
[M-H]
%
705.37
706.38
314.91 354.88
433.88 449.35 489.63 552.05
637.36
686.49
707.37
738.37
0
300 350 400 450 500 550 600 650 700 750 800
m/z
A mediolanus
E coli clone
idi-YAm fragment

FIG. 4

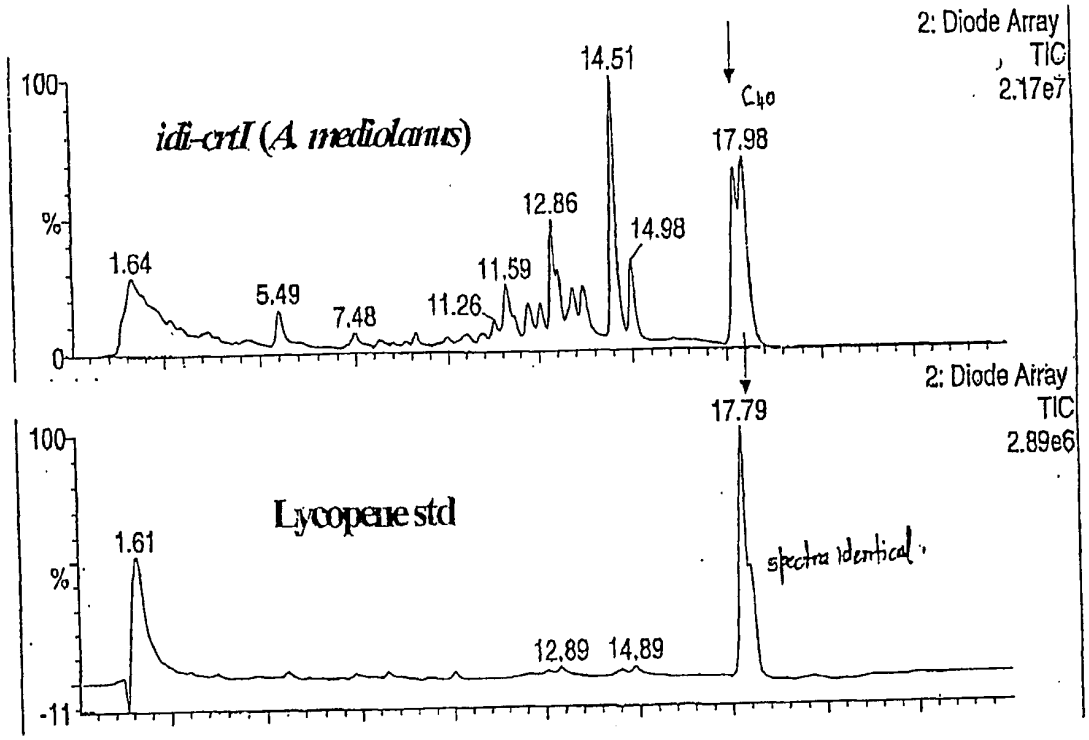

2: Diode Array
TIC
2.17e7
100
%
0
idi-crtI (A. mediolanus)
1.64
5.49
7.48
11.26
11.59
12.86
14.51
14.98
C40
17.98
2: Diode Array
TIC
2.89e6
17.79
100
%
-11
Lycopene std
1.61
12.89
14.89
spectra identical

FIG. 5

Lycopene std peak and shoulder

Doublet peak in *idi-crtI* clone

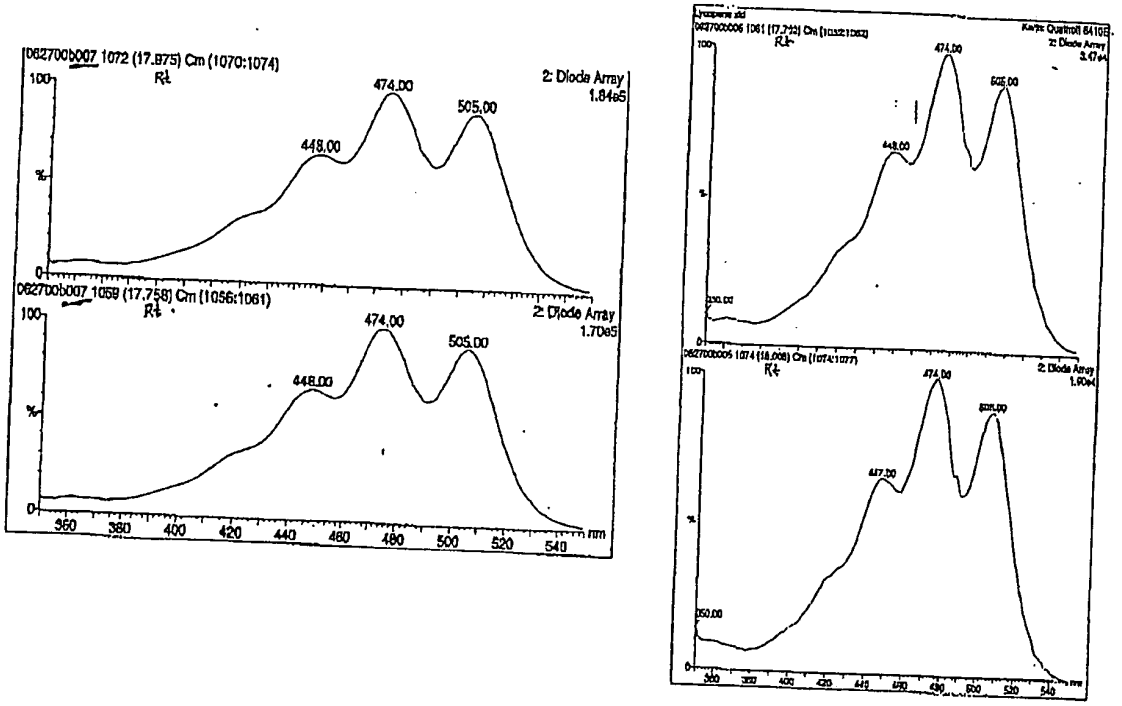


FIG. 6

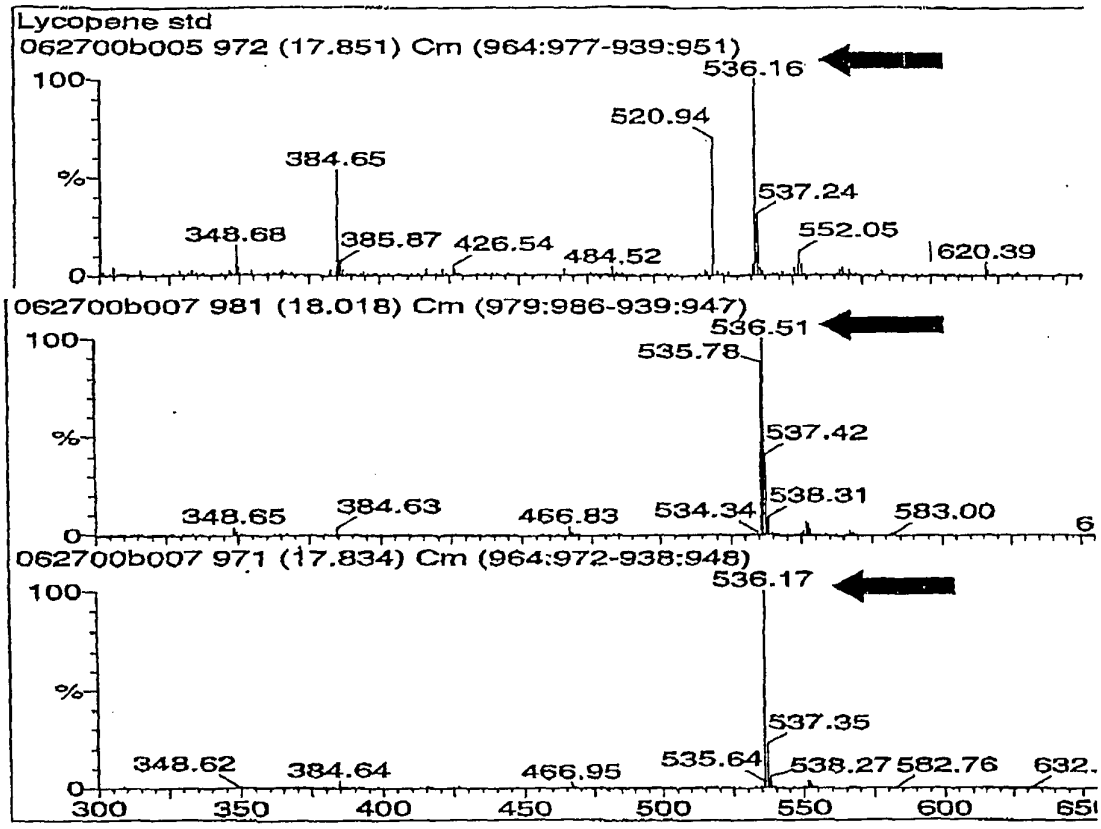

Lycopene std
062700b005 972 (17.851) Cm (964:977-939:951)
100
%
0
536.16
520.94
384.65
537.24
348.68
385.87
426.54
484.52
552.05
620.39
062700b007 981 (18.018) Cm (979:986-939:947)
100
%
0
536.51
535.78
537.42
348.65
384.63
466.83
534.34
538.31
583.00
062700b007 971 (17.834) Cm (964:972-938:948)
100
%
0
536.17
537.35
348.62
384.64
466.95
535.64
538.27
582.76
632.
300
350
400
450
500
550
600

FIG. 7

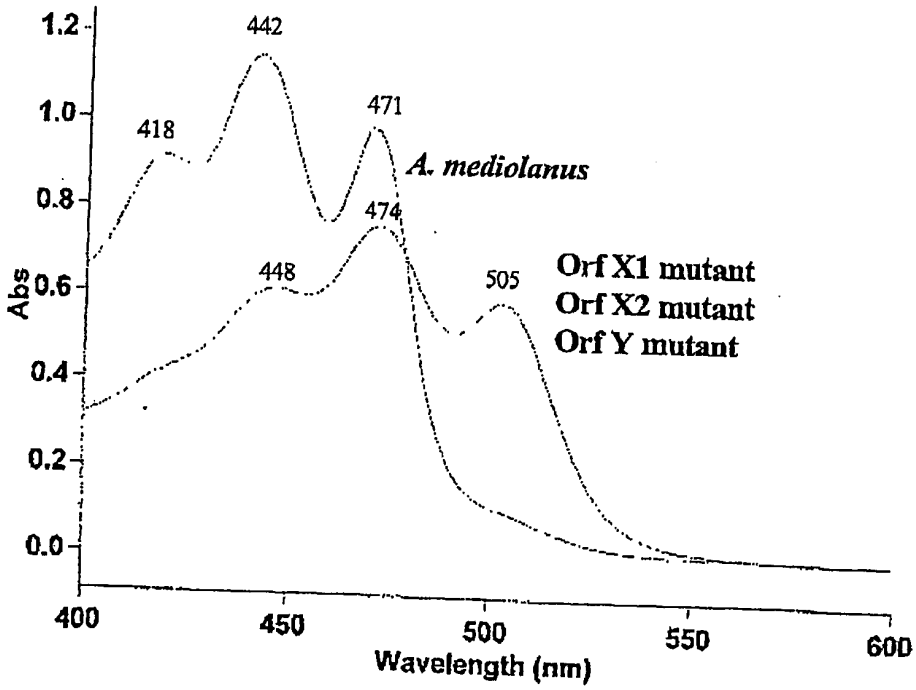

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs
400
450
500
550
600
Wavelength (nm)
418
442
471
474
448
505
A. mediolanus
Orf X1 mutant
Orf X2 mutant
Orf Y mutant

FIG. 8

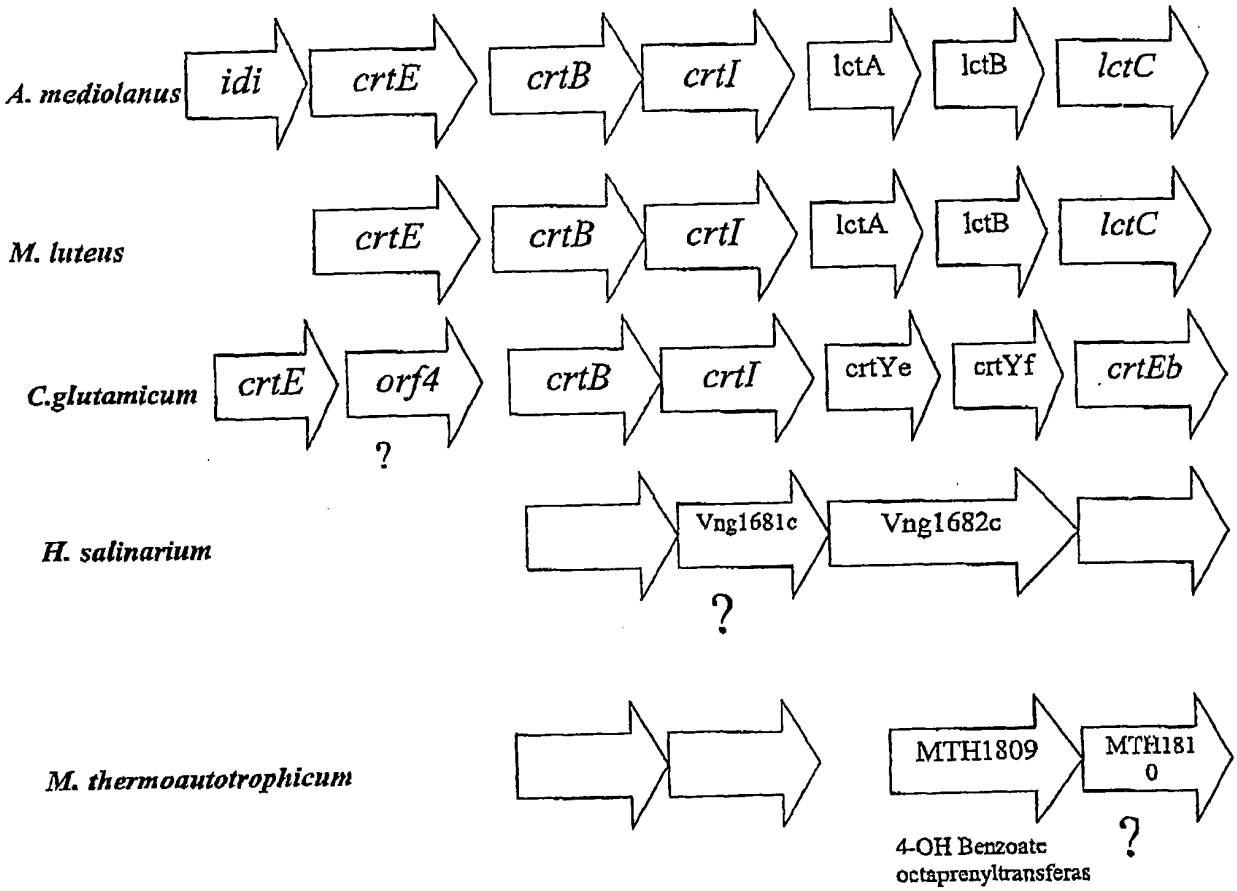

A. mediolanus
idi
crtE
crtB
crtI
lctA
lctB
lctC
M. luteus
crtE
crtB
crtI
lctA
lctB
lctC
C.glutamicum
crtE
orf4
?
crtB
crtI
crtYe
crtYf
crtEb
H. salinarium
Vng1681c
Vng1682c
?
M. thermoautotrophicum
MTH1809
MTH181
0
4-OH Benzoate
octaprenyltransferas
?

FIG. 9

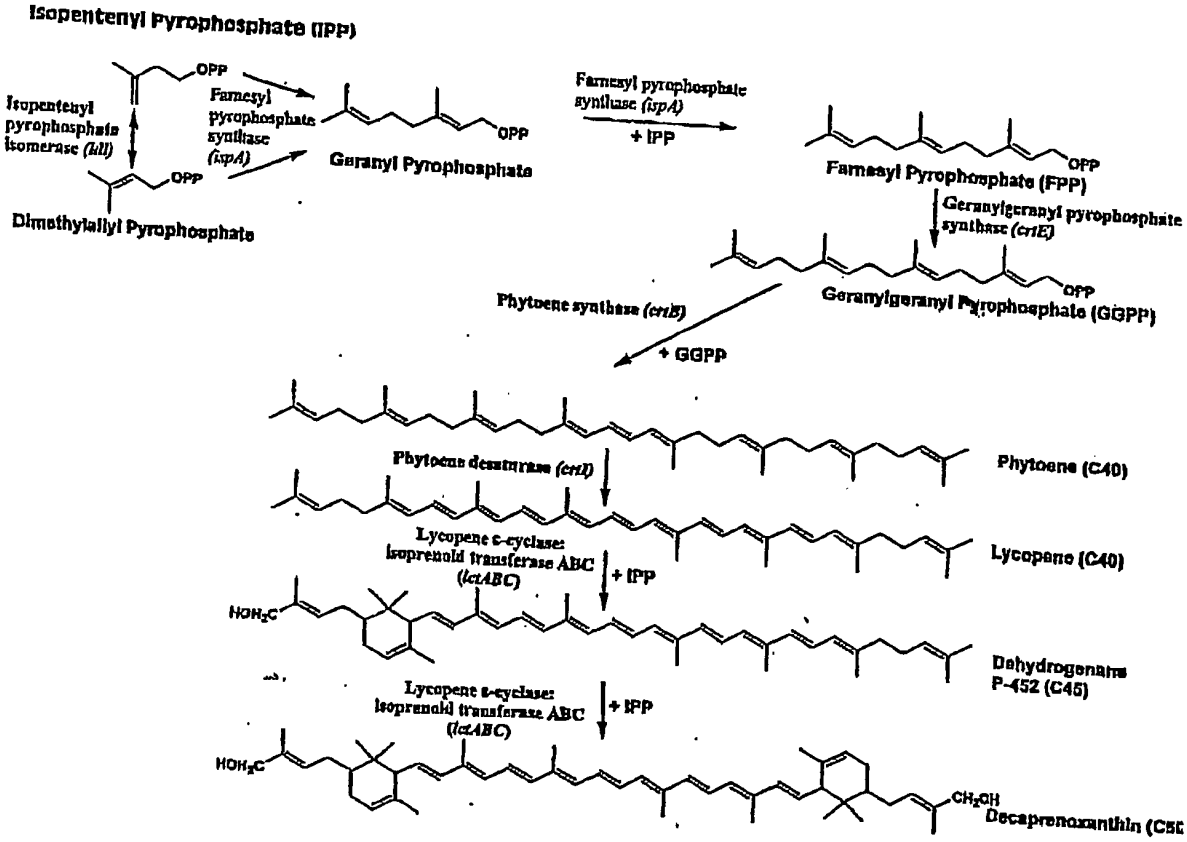
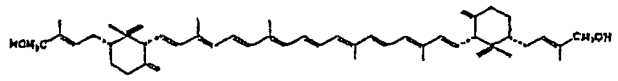

Isopentenyl Pyrophosphate (IPP)
OPP
Isopentenyl pyrophosphate isomerase (idi)
Farnesyl pyrophosphate synthase (ispA)
Geranyl Pyrophosphate
Dimethylallyl Pyrophosphate
Farnesyl pyrophosphate synthase (ispA)
+ IPP
Farnesyl Pyrophosphate (FPP)
Geranylgeranyl pyrophosphate synthase (crtE)
Geranylgeranyl Pyrophosphate (GGPP)
Phytoene synthase (crtB)
+ GGPP
Phytoene desaturase (crtI)
Phytoene (C40)
Lycopene (C40)
Lycopene ε-cyclase: Isoprenoid transferase ABC (lcrABC)
+ IPP
HOH2C
Dehydrogenase P-452 (C45)
CH2OH
Decaprenoxanthin (C50

FIG. 10

new structure for Sarcinaxanthin:
(2R,6S,2'R,6'S)-2,2'-Bis(4-hydroxy-3-methyl-2-butenyl)-γ,γ-carotene

$C_{50}H_{72}O_2$



Sarcina lutea (*Micrococcus luteus*)

447

Decaprenoxanthin, Dehydrogenase-P 439, ("Sarcinaxanthin"[2408,1474, 1473,957]; (2R,6R,2'R,6'R)-2,2'-Bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene

$C_{50}H_{72}O_2$ *Agromyces mediolanus*

456

bacterioruberin, α-Bacterioruberin, "Didemethylated spirilloxanthin" [2017,1458,1453,1432,1240,1239]; (2S,2'S)-2,2'-bis(3-hydroxy-3-methylbutyl)-3,4,3',4'-tetradehydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene-1,1'-diol

Halobacterium salinarium

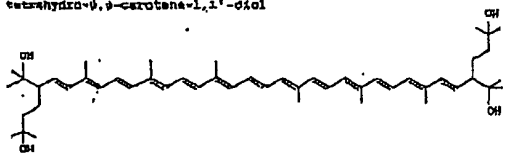


FIG. 11

ATCC 383 *Micrococcus luteus* CS0-carotenoid producing operon

Proposed translational start codons (bolded)

crtE (GGPP synthase)	688
crtB (phytoene synthase)	1788
crtI (phytoene desaturase)	2780
lctA (having homology with lctA of *A. mediolanus*)	4411
lctB (having homology with lctB of *A. mediolanus*)	4755
lctC (having homology with lctC of *A. mediolanus*)	5243

Proposed translational stop codons (underlined)

crtE (GGPP synthase)	1789
crtB (phytoene synthase)	2781
crtI (phytoene desaturase)	4409
lctA (having homology with lctA of *A. mediolanus*)	4756
lctB (having homology with lctB of *A. mediolanus*)	5247
lctC (having homology with lctC of *A. mediolanus*)	6116

```
   1  ctgccccccgc tgctcgtgca cgccatccgg ttcggcggcg gctacggggg tgcggtggtg
  61  cgggccctgc gccagctcgg gtgacccccgc ccgtggttgg acaggacccg ccgctgtcca
 121  gcatgatggt tattagaatt tctagtagtt acgaggcggg agtcaccggg tgacggagac
 181  cggagcgtgg agtgcgagcg tgagcccgca gtcgcgcgcg ctgcgtcggc tggtgcggct
 241  gaacgagggg atcgggtacc agatccgccg cctcatgggc ctgaaggaaa ccgactactc
 301  cgccatggcc ctgctcttgc ggagtccgat ggggcccacc gacctggccc acgctctgca
 361  catcaccacc gcttccgcca cggccgtggt ggaccggctc gcacgggccg gtcacgtggt
 421  gcgtgaaccg cacggagagg accgccgccg catgaccgtg cgggccgtgg ccggatcccg
 481  tgagcaggtg cgggagcacg tggtgcccat gatggacatg gtcgaggagg agctcgccgcg
 541  gctggacgag tccggccgcg gggccgtcct gcagttcctc accggcaccg ccgaccgcat
 601  ggaggactac ctggcgggtc tgcgcgaacg cccggccggc actggcggcg ccacccaggg
 661  catgcccggc cccgggggcgg agcgcccatg acctcggaga cagacaccgc ggcggatccc
 721  accgcggtct gggatgtgtt ccgcgcggcc gttgaccggg agctggacga gttcttcgac
 781  tccccgcgca acagggttcc ctacagcccg ggcttccccgg tgatgtggga tcgcatccgg
 841  cagcaggtgg tgggcggcaa gctgatccgg ccccgtctga cgcagatcgc gtggcgctcg
 901  ttcgccggtg agtcgagcac tgactccggc cgagaggccg agtgcgtgcg cctggcggcg
 961  tcgttcgaga tgctgcacgc ggcgctgatc gtgcacgacg acgtcgtgga ccgggactgg
1021  cgccgtcgtg ggcggcccac ggtgggcgag ctcttccgcc gcgacgcggt gcaggcgggg
1081  gccccgagg gcgaggccga gcacgcgggg gagtccgcgg cgatcctcgc gggagacctg
1141  cttctggcgg gtgcgctgcg gctggcgaac acgtgcaccg aggacccggg gcgggacgt
1201  gccgtggcag acgtggtctt cgaggcggtg accgcgtccg cggccggtga gctggacgac
1261  ctcctgctct ctctgcaccg ctacggcgcg gagcacccgg gcgtgcagga catcctggac
1321  atggagcggc tgaagaccgc cacgtactcg ttcgaggcac ccctgcgcgc cggcgccctg
1381  ctcgcgggag cgcccgagga gcaggcccag cgcctggcgc gggccggcgc ccagctcggg
1441  gtggcctacc aggtcgtcga cgacgtcctg ggaaccttcg gcgaccccga gctcaccggc
1501  aagtcggtgg acgccgatct gaactcgggc aaggccaccg tgctcaccgc ccacggaatg
1561  cagacccccg cggtgcggga cgtcctcgcg gagctcgcgg ccgggcgtac cacggtcgcc
1621  tccgcgcggg ctgccctgac ggcgtcgggа gcgcaggagg cagccgtggc agtggccacg
1681  gacctcgtgg accgggcccg ggccaccctg gacggtctcc cgctgcccgc tgcccagcgc
1741  gcggagctcg acgcgctgtg ccaccacgtc ctgaacagag actcgtagtg aggacccccа
1801  ccatgcccca ggacgcaccg gccgacgcgc cgctgagcct ctacaccgcc accgcgctgg
1861  cggcctcggg cgcggtgatc gggcgctact ccacgtcctt ctcgctggcg tgccggaccc
1921  tgccggcggc ggtgcgccgg gacatcgcgg ggatctacgc cctcgtgcgc gtggcggacg
1981  aggtggtgga cgggacggcc ggggcggcgg gtctcggcgc ggaccgggtg cgcgcggcgc
2041  tcgacgcgta cgaggccgag gtggcctccg cgctcgccac gggcttctcg accgacctgg
2101  tggtccacgg cttcgcgggc gtcgcccgcc gtcacggctt cggcacggag ctcacggagc
2161  cgttcttcgc gtccatgcgc gcggacctgg acgtggccga gcacgacggc gcctcgcttg
2221  agtcctacat ctacggctcg gcggaggtcg tggggctgat gtgcctggag gtcttcatgg
2281  acatgcccgg cacccgcgcc cagaccccgg agcagcgggа gatgctgcgc gccacggccc
```

```
2341  gccggctggg tgccgcgttc cagaaggtca acttcctgcg ggatctcggc gcggaccacg
2401  accagctcgg acgcacctac ttccccggcg cggaccctc ccacctggac gagacccgca
2461  agcggctgct gctcgcggac ctcggcgcgg acctggacgc ggccgtgccc gggatcctcg
2521  cgctggaccg ccgtgccggg cgcgcggtgc tgatcgcgca cggactgttc ggtgagctcg
2581  cacggcggat cgaggaggtg cccgcggcgg agctcacacg acggcgcatc agcgtgcccg
2641  ccggggtgaa gctgcggatc gccgcgagag cgctgtccgt caccgcgcgc acgggctcac
2701  acgggcgggg ccgagcccta gagtcggggc ccccggtgcc ggcggccgtg cccgaaacct
2761  cccggacggg ggccacccga tgacgcgcac ggtggtgatc ggcggcggct tcgcgggcct
2821  ggccacggcg ggcctgctcg cccgggacgg gcacagcgtc accctgctcg agcagcagga
2881  cacggtgggc ggccgctccg ggcggtggtc cgcggagggc ttctcgttcg acaccggacc
2941  cagctggtac ctcatgcccg aggtgatcga ccgctggttc accctgatgg gcacgagcgc
3001  cgccgagcag ctggacctgc gccggctgga ccagggctac cgcgtcttct tcgaggacca
3061  cctggcggaa ccgcccacgg acgtggtcac cggtcgtgcc gaggagctgt tcgagagcct
3121  cgacccggga tcctcccgcg cactgcgctc ctacctggac tcgggcgcgc aggtctacga
3181  gctcgccaag aagcacttcc tctacacgga cttcgcccac ctgctggacc ttgtgcgccc
3241  ggaggtgctc cgcaacctcc cgcggttggc aacgctgctg ggcacgtcca tgaagaacta
3301  cgttgcgcgc cgttttccgg agccgcggca gcgccagatc ctgggctacc ccgccgtctt
3361  cctgggggcg tcccctcgt ccgccccggc catgtaccac ctcatgagcc acctggacct
3421  caccgacgga gtgcagtacc cggtgggcgg gttcgccgcg ctggtggacg ccatggaacg
3481  gctcgtgcgc gaggccggcg tggagatcgt cacgggagcc accgtgaccg gcatcgaggt
3541  ggctcccgag ccgcggtcgc cgcgttcccg gttggccgca gcccgggcac gacgtcgcac
3601  cgccggcacg gtcacgggcg tcaccttccg cacggcgccg ggggcggacc cggggacgga
3661  gccgggcggc gtcgtcgccg gtgcggaggt caccgtgccc gcggacgtcg tcgtcggcgc
3721  cgcggacctg caccacctcc agaccccgcct gcttcccggc ccgttccgcg caccggagtc
3781  ccgctggaag cgccgcgacc ccgggccctc cggggtgctc gtgtgcctgg gcgtgcgcgg
3841  gaagctgccg cagctggccc accacaacct gctgttcacc gcggactggg atgagaactt
3901  cgggcgcatc gagtccggtg cggacctggc cgaggagacc tcgatctacg tgtccatgac
3961  gtcggcgacg gatcccggca ccgcgcccga gggggacgag aacctgttca tcctggtgcc
4021  ctgcccgcg gcacccgagt ggggtcacgg cggaaccacc gccccgggcg tcgacgagcc
4081  cggctccgcg caggtggagc gggtcgctga cgccgccatc gcgcagctcg cgcgctgggc
4141  gcagatcccg gacctggcct cgcggatcgt ggtgcgcagg acctacgggc ccgaggactt
4201  cgcggtgggg gtcaacgcgt ggcgcggctc cctgctgggc cccggacaca ttctgacgca
4261  gtccgcgatg ttccgtccca gcgtcaccga ccgtgggatc cgggggctgt tctacgccgg
4321  gtcctcggtg cgcccgggga tcggcgtgcc catgtgcctg atctcctccg aggtggtgcg
4381  ggacgccgtg cgggagagcg gggcgcgctg atgtacctgc tcctgctgct cgtcctcctg
4441  ggctgtttcg cgctcatcga ccggcgctgg aacctgtact tctggtccgg acacccgctg
4501  cgggcctggc tcgtgctggt caccgggggtg gtgttcttcc tcgcgtgggga cctggtgggg
4561  atcgccaacg gactgttctg gcacggcgag aactccctga ccctggggat cttcgtggct
4621  cccgagctgc ccctggaaga ggtcttcttc ctcgcgttcc tctgctacca gaccatggtc
4681  tacgtgctcg gcgcgcccgt gctgtggcgg tggctgaggg cccgcaccgg cgcggcacac
4741  gcggggaggc gggcatgacg tactggggcg tgaacgcggt cttcctgggg atggcggcgg
4801  tcgtgctgct gacgacggcg ctcgtgcggc gcccacccgc ccggttctgg ggagcgctcg
4861  cggcctccac agtgctgctc gtggtgctca ccgccgtctt cgacaacgtc atgatcgcct
4921  ccgggatcat gacgtacacg gaccgcaaca tctcgggcgt gcggatcggg ctcgccccgc
4981  tggaggactt cgcctaccgc gtggccggtg tgctgctgct gccgacgatg tggctgctgc
5041  tgggaggcac gccccgggcg gcggccggtg acgggcgggc gacggcggcg tcgtcgtcct
5101  ccgcggtcgc agccgcaacc gcagccggcg cgggcgacga gaacgcgagc ggtgaggacg
5161  cggacaccga tggtacgagc accgggcgcg cacatgccgg gggcaggccc agtgggaacc
5221  ccgccgatgg aagggacgaa ccgtgctgag gacgctgttc tgggcctcgc gcccgctgag
5281  ctgggtgaac accgcctacc cgttcgcggc ggccgtgctg ctgacgggcg gtttgccctg
5341  gtggctcgtg gcgctggggg ccgtgttctt cctggtgccc tacaacctgg cgatgtacgg
5401  catcaacgac gtcttcgact acgagtcgga cctgcgcaac ccctgcaagg gcggcgtgga
5461  gggcgcggtg gtggatcgcg ccgcccagcg cggcgtgctg cgggcctcgt gcctgctgcc
5521  ggtgccgttc gtcgcggtgc tggcggggta cgggatcgtg accgggaacc tgctgtccgt
5581  gctggtgctg gcggtgagcc tgttcgcggt ggtcgcgtac tcgtgggcgg ggctgcgctt
5641  taaggagcgc ccgttcgtgg atgcgatgac ctccgccacc cacttcgtct cgcccgccgt
5701  ctacggactg gtgctcgcac gggcggactt cacggtgggg ctgtgggcgg tgctcgtggg
5761  cttcttcctg tggggcatgg cctcgcagat gttcggggcg gtgcaggacg tggtaccgga
5821  ccgtgagggt gggctggcct ccgtggccac cgtgctcggt gcgcgcccca ccgtgtggct
5881  cgcggcgggc ctctacgccc tcgcaggtgc cctgatgctg ctcgcccagt ggccgggtca
```

```
5941  gctcgcggcg ctgctcgcgg tgccgtacct ggtcaacgcg ctgcgcttcc ggggcgtcac
6001  ggacgaggac tccggccggg ccaacgccgg gtggaggacg ttcctgtggt tgaactacgc
6061  gaccggtttc ctggtcacga tgctgctgat ctggtgggcc cgggttcacg tgctgtgaac
6121  ggatgcccaa cgcccgggac cggtgcggcc cggcctggtg aggccccgcc tggtgcatgg
6181  cccgcggtct gcgtgccccgg ggctggcatc atgggcgcat gagccgatcg acgttcgcca
6241  ctcacaccgc ccgggtcaac gacacgcagc tcgcctacac ggacgagggg cagggtctgg
6301  cggtcgtgct gctgcacggc cacggctacg accgctccat gtgggacgcg cagatcccgg
6361  tgctcgttga ccagggatgg cgcgtgatcg ccccggacct gcgcggcttc ggagattcgg
6421  aagtcacgcc gggcatcgtc tacaccgagg agttcgcggc ggacaccatc gcgctgctgg
6481  accgcctggg cctggactca gtggtgctgg tggggttttc gatggcgggg caggtggccc
6541  tgcagattgc tgcgacccac cctgagcggg tggccgcgct ggtcgtcaac gacacggtgc
6601  cgcacgccga gaacgcggcg gggcggcgtc gtcgtcacgt gggcgcggac gggatcctga
6661  cgggcgggat gccggcctac gcggacaggg tgctcgcctc catgatccgc gaggacaacg
6721  tggaacggct gcctgtggtg gccgacacgg tgcgcgagat gatcgccgcg tgtccggcgg
6781  agggggcggc cgcggccatg cgcgggcgtg ccgagcgcaa cgacttcacc gagacgctgc
6841  gggcgtggcg caagcccgcg ctcgtggtcg tgggggacgg ggacgcgttc gacggcggcg
6901  cggcccggcg gatggccgag ctgctgccgc acggcgagct c
```

FIG. 12

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.
SEQ NO: 01 is the nucleic acid sequence for the *lctA* gene isolated from *A. mediolanus*.

```
  1  atgaccttcc tccacctggg gctgctgctc gcctcgatcg cgtgcatcgc
 51  gctcgtcgac gcgcgctacc ggctgttctt ctggcgggcg ccgctgcggg
101  cgacggtcgt ggtcgccctc ggcgtcgcga tgctcctcgt ctgggacctc
151  tggggcatct cgctcggcat cttcttccgc gagccgaatg cctactcgac
201  ggggctgctc attgcgccgc acctgccgat cgaggagccg gtgttcctcg
251  ccttcctctg ccagctcgcg atggtcggct acacgggact gctgcgcctc
301  ctcgcgcacc gatccgcgca gcccgccacc ggccccgctg ccgactccac
351  cgccgaaggg gcccgccgat ga
```

SEQ NO: 02 is the nucleic acid sequence for the *lctB* gene isolated from *A. mediolanus*.

```
  1  atgagctacg ccgtgctctg cctcccgttc ctcgccgtct cggcggtgct
 51  cgccgcgatc gcctggcgac gtgctccggc cggtcacgcg gccgcgctcg
101  cgctcacggc gggcggcctc gtgctcctca ccgcggtgtt cgactcgctg
151  atgatcgccg cgggcctgtt cgactacgcc gacgcgcccc tgctcggccc
201  gcgcctcggg ctcgccccga tcgaggactt cgcctacccg atcgccgcgc
251  tgctgctctg ctccacggtc tggacgctgc tcgggcgagc ggatgcctcg
301  gcggctcgtg accggcccgc ccgcgcgccc agaggagccg agcgatga
```

SEQ NO: 03 is the nucleic acid sequence for the *lctC* gene isolated from *A. mediolanus*.

```
  1  atgagcgccg tcggcgccga ggcatccggc cagcgcctgc tccccgcgct
 51  cttcaccgca tcgcgcccgc tgagctggat caacaccgcc ttcccgttcg
101  cggccgcgta cctgctgacc gtgcgcgagg tcgacgtcgc gctcgtcgtc
151  ggcaccctgt tcttcctcgt gccgtacaac ctcgcgatgt acggcatcaa
201  cgacgtcttc gacttcgagt ccgacgcgcg gaatccgcgc aagggcggcg
251  tcgagggggc cctgctgccg cccgcccggc atcgcgcggt gctgatcgcc
301  gcggtggccc tgacggtgcc gttcgtcgtc tggctcgtgc tgctcggcgg
351  cccgtggtcg tgggcctggc tcgcgctcag cctgttcgcc gtggtggcgt
401  actcggcgcc gggcctcagg ttcaaggaga tcccggggcc tgactccctc
451  acctcgagca cgcacttcgt ctcgcccgcc tgctacgggc tcgccctcgc
501  gggggcgacg gtgacgccgc agctcgtgct gctgctgctc gcgttcttcg
551  tgtggggcgt cgcgagccac gccttcggcg cggtgcagga cgtcgtgccc
601  gatcgcgagg ccgggatcgg gtcgatcgcg accgcgctgg gggcccgccg
651  cacgacccgg ctcgcgatcg gcctctggct gctcgcgggc gtgctgatgc
701  tcggcacgtc gtggccgggg ccgctcgccg cggtactcgc cgtgccgtac
751  ctcgtcgcgg cgtggccgta ccgctcggtg agcgacgccg agtcggcgcg
801  cgcgaacggc ggctggcgct ggttcctcgc gatcaactac ggcgtcggct
851  tcgcggcgac gatgctgctg atctggtacg cgctgctcac ggcctga
```

SEQ NO: 04 is the amino acid sequence encoded by SEQ NO: 01.

```
  1  mtflhlglll asiacialvd aryrlffwra plratvvval gvamllvwdl
 51  wgislgiffr epnaystgll iaphlpieep vflaflcqla mvgytgllrl
101  lahrsaqpat gpaadstaeg arr
```

SEQ NO: 05 is the amino acid sequence encoded by SEQ NO: 02.

```
  1  msyavlclpf lavsavlaai awrrapagha aalaltaggl vlltavfdsl
 51  miaaglfdya dapllgprlg lapiedfayp iaalllcstv wtllgradas
101  aardrparap rgaer
```

SEQ NO: 06 is the amino acid sequence encoded by SEQ NO: 03.

```
  1  msavgaeasg qrllpalfta srplswinta fpfaaayllt vrevdvalvv
 51  gtlfflvpyn lamygindvf dfesdarnpr kggvegallp parhravlia
101  avaltvpfvv wlvllggpws wawlalslfa vvaysapglr fkeipgpdsl
151  tssthfvspa cyglalagat vtpqlvllll affvwgvash afgavqdvvp
201  dreagigsia talgarrttr laiglwllag vlmlgtswpg plaavlavpy
251  lvaawpyrsv sdaesarang gwrwflainy gvgfaatmll iwyallta
```

SEQ NO: 07 is the nucleic acid sequence for the *lctA* gene isolated from *M. luteus*.

```
  1  atgtacctgc tcctgctgct cgtcctcctg ggctgtttcg cgctcatcga
 51  ccggcgctgg aacctgtact tctggtccgg acacccgctg cgggcctggc
101  tcgtgctggt caccggggtg gtgttcttcc tcgcgtggga cctggtgggg
151  atcgccaacg gactgttctg gcacggcgag aactccctga ccctggggat
201  cttcgtggct cccgagctgc ccctggaaga ggtcttcttc ctcgcgttcc
251  tctgctacca gaccatggtc tacgtgctcg gcgcgcccgt gctgtggcgg
301  tggctgaggg cccgcaccgg cgcggcacac gcggggaggc gggcatga
```

SEQ NO: 08 is the nucleic acid sequence for the *lctB* gene isolated from *M. luteus*.

```
  1  atgacgtact ggggcgtgaa cgcggtcttc ctggggatgg cggcggtcgt
 51  gctgctgacg acggcgctcg tgcggcgccc acccgcccgg ttctggggag
101  cgctcgcggc ctccacagtg ctgctcgtgg tgctcaccgc cgtcttcgac
151  aacgtcatga tcgcctccgg gatcatgacg tacacggacc gcaacatctc
201  gggcgtgcgg atcgggctcg ccccgctgga ggacttcgcc taccccgtgg
251  ccggtgtgct gctgctgccg acgatgtggc tgctgctggg aggcacgccc
301  ggggcggcgg ccggtgacgg gcgggcgacg gcggcgtcgt cgtcctccgc
351  ggtcgcagcc gcaaccgcag ccggcgcggg cgacgagaac gcgagcggtg
401  aggacgcgga caccgatggt acgagcaccg ggcgcgcaca tgccgggggc
451  aggcccagtg ggaaccccgc cgatggaagg gacgaaccgt gctga
```

SEQ NO: 09 is the nucleic acid sequence for the *lctC* gene isolated from *M. luteus*.

```
  1  gtgctgagga cgctgttctg ggcctcgcgc ccgctgagct gggtgaacac
 51  cgcctacccg ttcgcggcgg ccgtgctgct gacgggcggt ttgccctggt
101  ggctcgtggc gctgggggcc gtgttcttcc tggtgcccta caacctggcg
```

```
151  atgtacggca tcaacgacgt cttcgactac gagtcggacc tgcgcaaccc
201  ccgcaagggc ggcgtggagg gcgcggtggt ggatcgcgcc gcccagcgcg
251  gcgtgctgcg ggcctcgtgc ctgctgccgg tgccgttcgt cgcggtgctg
301  gcggggtacg ggatcgtgac cgggaacctg ctgtccgtgc tggtgctggc
351  ggtgagcctg ttcgcggtgg tcgcgtactc gtgggcgggg ctgcgcttta
401  aggagcgccc gttcgtggat gcgatgacct ccgccaccca cttcgtctcg
451  cccgccgtct acggactggt gctcgcacgg gcggacttca cggtggggct
501  gtgggcggtg ctcgtgggct tcttcctgtg gggcatggcc tcgcagatgt
551  tcggggcggt gcaggacgtg gtaccggacc gtgagggtgg gctggcctcc
601  gtggccaccg tgctcggtgc gcgccccacc gtgtggctcg cggcgggcct
651  ctacgccctc gcaggtgccc tgatgctgct cgcccagtgg ccgggtcagc
701  tcgcggcgct gctcgcggtg ccgtacctgg tcaacgcgct gcgcttccgg
751  ggcgtcacgg acgaggactc cggccgggcc aacgccgggt ggaggacgtt
801  cctgtggttg aactacgcga ccggtttcct ggtcacgatg ctgctgatct
851  ggtgggcccg ggttcacgtg ctgtga
```

SEQ NO: 10 is the amino acid sequence encoded by SEQ NO: 07.

```
  1  myllllllvll gcfalidrrw nlyfwsghpl rawlvlvtgv vfflawdlvg
 51  ianglfwhge nsltlgifva pelpleevff laflcyqtmv yvlgapvlwr
101  wlrartgaah agrra
```

SEQ NO: 11 is the amino acid sequence encoded by SEQ NO: 08.

```
  1  mtywgvnavf lgmaavvllt talvrrppar fwgalaastv llvvltavfd
 51  nvmiasgimt ytdrnisgvr iglapledfa ypvagvlllp tmwlllggtp
101  gaaagdgrat aassssavaa ataagagden asgedadtdg tstgrahagg
151  rpsgnpadgr depc
```

SEQ NO: 12 is the amino acid sequence encoded by SEQ NO: 09.

```
  1  vlrtlfwasr plswvntayp faaavlltgg lpwwlvalga vfflvpynla
 51  mygindvfdy esdlrnprkg gvegavvdra aqrgvlrasc llpvpfvavl
101  agygivtgnl lsvlvlavsl favvayswag lrfkerpfvd amtsathfvs
151  pavyglvlar adftvglwav lvgfflwgma sqmfgavqdv vpdregglas
201  vatvlgarpt vwlaaglyal agalmllaqw pgqlaallav pylvnalrfr
251  gvtdedsgra nagwrtflwl nyatgflvtm lliwwarvhv l
```

SEQ NO: 13 is the nucleic acid sequence for the *idi* gene isolated from *A. mediolanus*.

```
  1  atgaccgacc tcagcatcac gccgctgccg gcccaggccg caccggtgca
 51  gcccgcatcc agcgccgaat tggtcgtgct gctcgacgag gccggcaacc
101  agatcggcac cgccccgaag tcgagcgtgc acggcgccga caccgccctc
151  catctcgcgt tctcctgcca cgtcttcgac gacgacggcc gcctcctggt
201  gacccgtcgc gcgctcggca aggtcgcctg gcccggcgtg tggaccaact
251  ccttctgcgg gcaccccgcc ccggccgagc cgctgccgca cgcggtgcgc
301  cgccgggccg agttcgagct cggcctcgag ctccgcgacg tcgagccggt
351  gctgccgttc ttccgctacc gggcgacgga tgcctcgggc atcgtcgagc
401  acgagatctg cccggtctac acggcgcgca caagctcggt gccggcgccg
451  catcccgacg aggtcctcga cctcgcctgg gtcgaaccgg gcgagctcgc
501  caccgcggtc cgcgccgcgc cctgggcgtt cagtccctgg ctcgtgctgc
551  aggcgcagct gctgcccttc ctcggcggcc acgccgacgc gcgcgtccgc
601  acggaagcgc tcgtctcgtg a
```

SEQ NO: 14 is the nucleic acid sequence for the *crtE* gene isolated from *A. mediolanus*.

```
   1  gtgagcctcg tcgcgaccgt ggtcgccccg agccggcagg cggaggtgga
  51  gcgctacctc ggcggcttct tcgacgacgc catcgtgcgg gccgacgcgc
 101  acgccgccga ctaccggcgg ctctgggcgg cggcgcggga cgccgcgagc
 151  ggcggcaagc ggatccgccc caggctcgtg ctgggcgcct acgacgcgct
 201  cgccgcgcag ggtgcgccgg cgagcggccg cgaacgggcc gacgccgagc
 251  cggccgccgc cgcggaggcc gtggcgctcg cggcggcctt cgagctgctg
 301  cacaccgcgt tcctcgtgca cgacgacgtc atcgaccgcg acctcgtgcg
 351  ccggggcgag cccaacgtcg ccggccgctt cgcgctcgac gccgcgctgc
 401  gcgggctcga gcgggagcgg gcggacgcct acggccaggc ctcggcgatc
 451  ctcgcgggcg acctgctgat cgcggcggcg cactccgtgg cggccgcctc
 501  gacgtgccgg tcgagcgccg gcgagccatc ctcgccgtcc ttgacgaagt
 551  gcgtcttcgc cgccgccgcg ggcgagcacg ccgacgtccg gcacgccgcc
 601  ggggtgcggc ccggggaggc ggacatcctc gcgatgatcg aggacaagac
 651  ggcctgctac tcgttcagcg cgccgctccg ggcgggcgcg ctgctcgccg
 701  gcgccccgcg cgcgacggtc gaacggctcg gcgagatcgg ccgtcgactc
 751  ggcgtcgcct tccagctgca ggacgacgtg ctcggcgtct acggcgacga
 801  gcgggtgacc ggcaagacgg cgctcgggga cctccgcgag ggcaaggaga
 851  cgctgctcat cgcctacgcg cgggggcacg cggcctgggt cgcggcatcc
 901  ggcgccttcg gccggcccga cctcgacgag gcgggcgccc gccccctccg
 951  cgcggcgatc gaggcgagcg gcgcccgcgc ccgcgtcgag gcgcgcatcg
1001  ccgaggaggc ggccgcggcg cgcacggcga tcgccgcggc gggcctgccc
1051  gccgcgctcg aagccgagtt gctcggcctc gccgccgaag ccaccaggag
1101  gtcgaggtga
```

SEQ NO: 15 is the nucleic acid sequence for the *crtB* gene isolated from *A. mediolanus*.

```
   1  gtgagcacgc gcaccaccca gcgcacgacc gcgccgcccg caccgtccac
  51  cggcctcgcc ctctacgacc gcaccgccgc cgagggctcg gcccgggtca
 101  tccgggcgta ctcgacctcc ttcggcctcg cgagccggct ctgctccccc
 151  gccgtccgcg agcacctcgc cgaggtctac gcgctcgtgc gcatcgccga
 201  cgagctcgtc gacggcccgg ccgaggaggc cgggctgccg tgcgagcgcc
 251  gccgcgagct gctcgacgcc ctcgaggccg acacggaggc cgccttcgag
 301  agcggctaca gcgccaacct cgtggtgcac gccttcgcgc gcgcggcgcg
 351  gcgcagcggc ttcggccagg agctcacccg gcccttcttc gcctcgatgc
 401  gacgcgacct cgagcccatc gccttcaccg aggagcgcga gctcgacgaa
 451  tacgtctacg gctcggccga ggtcgtcggc ctgatgtgcc tgcgcggctt
 501  cgcgatcggg ctcgcccccg acgccgagcg cgacgcccgc tgggagcgcg
 551  gcgcgcgggc gctgggctcg gcgttccagc gggtcaactt cctgcgggac
 601  ctcggggagg atgcctcgct ccgcggacgc cgctacttcc cgggcgtcga
 651  tccggtgagc ttctcggagg cccagcaact gcgcctcctc gacggcatcg
 701  acgcggagct cgacgaggcg gccgccgtga tcccggagct gccccgcggc
 751  tgccgcgtcg cggtcgccgc ggcgcacggc ctgttcggcg agctctccgc
 801  ccggctccgc cgcacgcccg cggccgagct cgtcacccgg cgggtccggg
 851  tgcccgcgcc gcgcaagctc gccatcgtca cccgcgtggt cgcccgcgga
 901  ggccggccgt ga
```

SEQ NO: 16 is the nucleic acid sequence for the *crtI* gene isolated from *A. mediolanus*.

```
   1  gtgagccgcg cggtcgtcat cggcggcggc atcgccgggc tcgccacggc
  51  ggcgctgctc gcccgcgacg ggcacgaggt gcggctcttc gaggcgcgcg
 101  acgagctcgg cggccgtgcc gggcgctggc gggcgaacgg cttcctgttc
 151  gacaccggtc cgagctggta cctcatgcca gaggtgttcg agcacttcta
 201  ccgcttgatg ggcaccacgg cggccgagga gctcgagctc gtgcgcctcg
 251  accccggcta ccgggtgtac ttcgagggct acgacgagcc ggtcgacgtg
 301  cgggccgagc gcgaggcatc catcgccctc ttcgagtcga tcgagccggg
 351  cgcgggcgcc gcgctcgccc ggcacctcga ctccgccaac gagacgtacc
 401  ggctcgcgat gacgcacttc ctctacaccg acttcgccca cccgggggcg
 451  ctgctcgccg cgccggtccg gcggcggctc ggccggctcg cgaagctgct
 501  gctcgaaccg ctcgaccgca tggtggggcg ctccttcgac gacgtgcggc
 551  tgcggcagat cctgggctac ccggcggtct tcctcggcac ctcgcccgag
 601  cgggcgccga gcatgtacca cctgatgagc cgcttcgacc tcgccgacgg
 651  ggtgttctac ccgatgggcg gcttcggcga gatcatcgcg agcgtggccc
 701  ggctggcccg gcgggccggg gccgagctcg tcaccggcgc gcgggtgctc
 751  ggcatcgaga cggccggcgg gcgcgccacg ggcgtgcgcg tgcagcacca
 801  cggcccgacc ggtggcaccg gcaccgagga gttcctggag gccgagctcg
 851  tcgtctccgc cgccgatctg caccacacgg atgccgagct gctcccgccc
 901  cgcgcgcgga cgcggagcga ggcatcctgg tcgcgccgcg accccggacc
 951  cggcgcggtg ctcgtcatgc tcggcgtgca cgggcggctg ccggagctcg
1001  cccaccacac gctctgcttc acggccgact ggcgcacgaa cttccagcgg
1051  gtgttcggct cgcgaccggc gatccccgac ccggcgtcgt tctacgtctg
1101  ccgcccgagt gcgacggatc cgggcgtggc gccccccggc tgcgagaacc
1151  tgttcctgct cgtgccggtg cccgccgacc ccacaatcgg cgccggcggt
1201  gtcgacggcc gcggcgaccg ggcggtcgag gagacggccg accgggcgat
1251  cgcgaccctc gccgagtggg ccggcatccc cgacctcgcc gagcggatcc
1301  tcgtgcgccg cacgatcggg cccgcggact tcgaggactg gttccagtcc
1351  tggcgcggct cggcgctcgg cccggggcac accctgcggc agagcgccat
1401  gttccggggg cgcacggcct cggcgaacgt cgaggggctg tacttcgcgg
1451  gggcgacgac gatcccgggc atcggcctgc cgatgtgcct gatcagcgcc
1501  gagctcgtcg cgaaggccgt gcgcggcgag gatgccccgg gcccgctccc
1551  ggagccgagc gaggagccgc acccagaccc gctgcaccca gacccgctgc
1601  acccagaccg gctcgaccgg gagcgcaccg gatga
```

SEQ NO: 17 is the amino acid sequence encoded by SEQ NO: 13.

```
   1  mtdlsitplp aqaapvqpas saelvvllde agnqigtapk ssvhgadtal
  51  hlafschvfd ddgrllvtrr algkvawpgv wtnsfcghpa paeplphavr
 101  rraefelgle lrdvepvlpf fryratdasg iveheicpvy tartssvpap
 151  hpdevldlaw vepgelatav raapwafspw lvlqaqllpf lgghadarvr
 201  tealvs
```

SEQ NO: 18 is the amino acid sequence encoded by SEQ NO: 14.

```
   1  vslvatvvap srqaeveryl ggffddaivr adahaadyrr lwaaardaas
  51  ggkrirprlv lgaydalaaq gapasgrera daepaaaaea valaaafell
 101  htaflvhddv idrdlvrrge pnvagrfald aalrglerer adaygqasai
 151  lagdlliaaa hsvaaastcr ssagepssps ltkcvfaaaa gehadvrhaa
 201  gvrpgeadil amiedktacy sfsaplraga llagapratv erlgeigrrl
 251  gvafqlqddv lgvygdervt gktalgdlre gketlliaya rghaawvaas
 301  gafgrpdlde agarplraai easgararve ariaeeaaaa rtaiaaaglp
 351  aaleaellgl aaeatrrsr
```

SEQ NO: 19 is the amino acid sequence encoded by SEQ NO: 15.

```
  1  vstrttqrtt appapstgla lydrtaaegs arviraysts fglasrlcsp
 51  avrehlaevy alvriadelv dgpaeeaglp cerrrellda leadteaafe
101  sgysanlvvh afaraarrsg fgqeltrpff asmrrdlepi afteerelde
151  yvygsaevvg lmclrgfaig lapdaerdar wergaralgs afqrvnflrd
201  lgedaslrgr ryfpgvdpvs fseaqqlrll dgidaeldea aavipelprg
251  crvavaaahg lfgelsarlr rtpaaelvtr rvrvpaprkl aivtrvvarg
301  grp
```

SEQ NO: 20 is the amino acid sequence encoded by SEQ NO: 16.

```
  1  vsravviggg iaglataall ardghevrlf eardelggra grwrangflf
 51  dtgpswylmp evfehfyrlm gttaaeelel vrldpgyrvy fegydepvdv
101  raereasial fesiepgaga alarhldsan etyrlamthf lytdfahpga
151  llaapvrrrl grlaklllep ldrmvgrsfd dvrlrqilgy pavflgtspe
201  rapsmyhlms rfdladgvfy pmggfgeiia svarlarrag aelvtgarvl
251  gietaggrat gvrvqhhgpt ggtgteefle aelvvsaadl hhtdaellpp
301  rartrseasw srrdpgpgav lvmlgvhgrl pelahhtlcf tadwrtnfqr
351  vfgsrpaipd pasfyvcrps atdpgvappg cenlfllvpv padptigagg
401  vdgrgdrave etadraiatl aewagipdla erilvrrtig padfedwfqs
451  wrgsalgpgh tlrqsamfrg rtasanvegl yfagattipg iglpmclisa
501  elvakavrge dapgplpeps eephpdplhp dplhpdrldr ertg
```

SEQ NO: 21 is the nucleic acid sequence for the *crtE* gene isolated from *M. luteus*.

```
   1  atgacctcgg agacagacac cgcggcggat cccaccgcgg tctgggatgt
  51  gttccgcgcg gccgttgacc gggagctgga cgagttcttc gactccccgc
 101  gcaacagggt tccctacagc ccgggcttcc cggtgatgtg ggatcgcatc
 151  cggcagcagg tggtgggcgg caagctgatc cggccccgtc tgacgcagat
 201  cgcgtggcgc tcgttcgccg gtgagtcgag cactgactcc ggccgagagg
 251  ccgagtgcgt gcgcctggcg gcgtcgttcg agatgctgca cgcggcgctg
 301  atcgtgcacg acgacgtcgt ggaccgggac tggcgccgtc gtgggcggcc
 351  cacggtgggc gagctcttcc gccgcgacgc ggtgcaggcg ggggcccccg
 401  agggcgaggc cgagcacgcg ggggagtccg cggcgatcct cgcgggagac
 451  ctgcttctgg cgggtgcgct gcggctggcg accacgtgca ccgaggaccc
 501  ggggcgggga cgtgccgtgg cagacgtggt cttcgaggcg gtgaccgcgt
 551  ccgcggccgg tgagctggac gacctcctgc tctctctgca ccgctacggc
 601  gcggagcacc cgggcgtgca ggacatcctg gacatggagc ggctgaagac
 651  cgccacgtac tcgttcgagg caccctgcg cgccggcgcc ctgctcgcgg
 701  gagcgcccga ggagcaggcc cagcgcctgg cgcgggccgg cgcccagctc
 751  ggggtggcct accaggtcgt cgacgacgtc ctgggaacct tcggcgaccc
 801  cgagctcacc ggcaagtcgg tggacgccga tctgaactcg ggcaaggcca
 851  ccgtgctcac cgcccacgga atgcagaccc ccgcggtgcg ggacgtcctc
 901  gcggagctcg cggccgggcg taccacggtc gcctccgcgc gggctgccct
 951  gacggcgtcg ggagcgcagg aggcagccgt ggcagtggcc acggacctcg
1001  tggaccgggc ccgggccacc ctggacggtc tcccgctgcc cgctgcccag
1051  cgcgcggagc tcgacgcgct gtgccaccac gtcctgaaca gagactcgta
1101  g
```

SEQ NO: 22 is the nucleic acid sequence for the *crtB* gene isolated from *M. luteus*.

```
   1  gtgaggaccc ccaccatgcc ccaggacgca ccggccgacg cgccgctgag
  51  cctctacacc gccaccgcgc tggcggcctc gggcgcggtg atcgggcgct
 101  actccacgtc cttctcgctg gcgtgccgga ccctgccggc ggcggtgcgc
 151  cgggacatcg cggggatcta cgccctcgtg cgcgtggcgg acgaggtggt
 201  ggacgggacg gccggggcgg cgggtctcgg cgcggaccgg gtgcgcgcgg
 251  cgctcgacgc gtacgaggcc gaggtggcct ccgcgctcgc cacgggcttc
 301  tcgaccgacc tggtggtcca cggcttcgcg ggcgtcgccc gccgtcacgg
 351  cttcggcacg gagctcacgg agccgttctt cgcgtccatg cgcgcggacc
 401  tggacgtggc cgagcacgac ggcgcctcgc ttgagtccta catctacggc
 451  tcggcggagg tcgtggggct gatgtgcctg gaggtcttca tggacatgcc
 501  cggcacccgc gcccagaccc cggagcagcg ggagatgctg cgcgccacgg
 551  cccgccggct gggtgccgcg ttccagaagg tcaacttcct gcgggatctc
 601  ggcgcggacc acgaccagct cggacgcacc tacttccccg gcgcggaccc
 651  ctcccacctg gacgagaccc gcaagcggct gctgctcgcg gacctcggcg
 701  cggacctgga cgcggccgtg cccgggatcc tcgcgctgga ccgccgtgcc
 751  gggcgcgcgg tgctgatcgc gcacggactg ttcggtgagc tcgcacggcg
 801  gatcgaggag gtgcccgcgg cggagctcac acgacggcgc atcagcgtgc
 851  ccgccggggt gaagctgcgg atcgccgcga gagcgctgtc cgtcaccgcg
 901  cgcacgggct cacacgggcg gggccgagcc ctagagtcgg ggcccccggt
 951  gccggcggcc gtgcccgaaa cctcccggac gggggccacc cgatga
```

SEQ NO: 23 is the nucleic acid sequence for the *crtI* gene isolated from *M. luteus*.

```
   1  atgacgcgca cggtggtgat cggcggcggc ttcgcgggcc tggccacggc
  51  gggcctgctc gcccgggacg ggcacagcgt caccctgctc gagcagcagg
 101  acacggtggg cggccgctcc gggcggtggt ccgcggaggg cttctcgttc
 151  gacaccggac ccagctggta cctcatgccc gaggtgatcg accgctggtt
 201  caccctgatg ggcacgagcg ccgccgagca gctggacctg cgccggctgg
 251  acccgggcta ccgcgtcttc ttcgaggacc acctggcgga accgcccacg
 301  gacgtggtca ccggtcgtgc cgaggagctg ttcgagagcc tcgacccggg
 351  atcctcccgc gcactgcgct cctacctgga ctcgggcgcg caggtctacg
 401  agctcgccaa gaagcacttc ctctacacgg acttcgccca cctgctggac
 451  cttgtgcgcc cggaggtgct ccgcaacctc ccgcggttgg caacgctgct
 501  gggcacgtcc atgaagaact acgttgcgcg ccgttttccg gagccgcggc
 551  agcgccagat cctgggctac cccgccgtct tcctgggggc gtcccctcg
 601  tccgccccgg ccatgtacca cctcatgagc cacctggacc tcaccgacgg
 651  agtgcagtac ccggtgggcg ggttcgccgc gctggtggac gccatggaac
 701  ggctcgtgcg cgaggccggc gtggagatcg tcacgggagc caccgtgacc
 751  ggcatcgagg tggctcccga gccgcggtcg ccgcgttccc ggttggccgc
 801  agcccgggca cgacgtcgca ccgccggcac ggtcacgggc gtcaccttcc
 851  gcacggcgcc ggggggcgac ccggggacgg agccgggcgg cgtcgtcgcc
 901  ggtgcggagg tcaccgtgcc cgcggacgtc gtcgtcggcg ccgcggacct
 951  gcaccacctc cagacccgcc tgcttcccgg cccgttccgc gcaccggagt
1001  cccgctggaa gcgccgcgac cccgggccct ccggggtgct cgtgtgcctg
1051  ggcgtgcgcg ggaagctgcc gcagctggcc caccacaacc tgctgttcac
1101  cgcggactgg gatgagaact tcgggcgcat cgagtccggt gcggacctgg
1151  ccgaggagac ctcgatctac gtgtccatga cgtcggcgac ggatcccggc
1201  accgcgcccg aggggacga gaacctgttc atcctggtgc cctcgcccgc
1251  ggcacccgag tggggtcacg gcggaaccac cgccccgggc gtcgacgagc
1301  ccggctccgc gcaggtggag cgggtcgctg acgccgccat cgcgcagctc
```

```
1351  gcgcgctggg cgcagatccc ggacctggcc tcgcggatcg tggtgcgcag
1401  gacctacggg cccgaggact tcgcggtggg ggtcaacgcg tggcgcggct
1451  ccctgctggg ccccggacac attctgacgc agtccgcgat gttccgtccc
1501  agcgtcaccg accgtgggat ccgggggctg ttctacgccg ggtcctcggt
1551  gcgcccgggg atcggcgtgc ccatgtgcct gatctcctcc gaggtggtgc
1601  gggacgccgt gcgggagagc ggggcgcgct ga
```

SEQ NO: 24 is the amino acid sequence encoded by SEQ NO: 21.

```
  1  mtsetdtaad ptavwdvfra avdreldeff dsprnrvpys pgfpvmwdri
 51  rqqvvggkli rprltqiawr sfagesstds greaecvrla asfemlhaal
101  ivhddvvdrd wrrrgrptvg elfrrdavqa gapegeaeha gesaailagd
151  lllagalrla ttctedpgrg ravadvvfea vtasaageld dlllslhryg
201  aehpgvqdil dmerlktaty sfeaplraga llagapeeqa qrlaragaql
251  gvayqvvddv lgtfgdpelt gksvdadlns gkatvltahg mqtpavrdvl
301  aelaagrttv asaraaltas gaqeaavava tdlvdrarat ldglplpaaq
351  raeldalchh vlnrds
```

SEQ NO: 25 is the amino acid sequence encoded by SEQ NO: 22.

```
  1  vrtptmpqda padaplslyt atalaasgav igrystsfsl acrtlpaavr
 51  rdiagiyalv rvadevvdgt agaaglgadr vraaldayea evasalatgf
101  stdlvvhgfa gvarrhgfgt eltepffasm radldvaehd gaslesyiyg
151  saevvglmcl evfmdmpgtr aqtpeqreml ratarrlgaa fqkvnflrdl
201  gadhdqlgrt yfpgadpshl detrkrllla dlgadldaav pgilaldrra
251  gravliahgl fgelarriee vpaaeltrrr isvpagvklr iaaralsvta
301  rtgshgrgra lesgppvpaa vpetsrtgat r
```

SEQ NO; 26 is the amino acid sequence encoded by SEQ NO: 23.

```
  1  mtrtvviggg faglatagll ardghsvtll eqqdtvggrs grwsaegfsf
 51  dtgpswylmp evidrwftlm gtsaaeqldl rrldpgyrvf fedhlaeppt
101  dvvtgraeel fesldpgssr alrsyldsga qvyelakkhf lytdfahlld
151  lvrpevlrnl prlatllgts mknyvarrfp eprqrqilgy pavflgasps
201  sapamyhlms hldltdgvqy pvggfaalvd amerlvreag veivtgatvt
251  gievapeprs prsrlaaara rrrtagtvtg vtfrtapgad pgtepggvva
301  gaevtvpadv vvgaadlhhl qtrllpgpfr apesrwkrrd pgpsgvlvcl
351  gvrgklpqla hhnllftadw denfgriesg adlaeetsiy vsmtsatdpg
401  tapegdenlf ilvpspaape wghggttapg vdepgsaqve rvadaaiaql
451  arwaqipdla srivvrrtyg pedfavgvna wrgsllgpgh iltqsamfrp
501  svtdrgirgl fyagssvrpg igvpmcliss evvrdavres gar
```

SEQ ID NOS: 27-30 are primers used to amplify regions of the Y1 operon.

AIDINDEF 5'- TTCATATGTCACTAGCCAGGCGAGATATCC-3'
APDHIIIR 5'- GAAAGCTTAAGAAGATGCCGAGCGAGATG-3'
AXHIIIR 5'- AGAAGCTTTGTACGGCACGAGGAAGAACAG –3'
AYHIIIR 5'- GAAAGCTTCTCCGTGACGAGATCCTGAG-3'

SEQ ID NOS: 31 and 32 are primers used to amplify ORFY.
AYPACF 5'-GTCTTAATTAACTGCTGCTCTGCTCCACGGTCT -3'
AYXBAR 5'- TATCTAGACGCTCCGTGACGAGATCCTGAG -3'

SEQ ID NOS: 33 is a primer used to amplify out the region of *Agromyces mediolanus* genomic DNA containing the X1, X2, and Y ORFs.
AXSPHF 5'-TAGGCATGCAACGTCGAGGGGCTGTACTTC -3'

SEQ ID NOS: 34 and 35 are primers used to amplify a mutated ORFX1, fragment.
X1A 5'-GCTCGTCGACGCGCGCTAGCCGGCTGTTCTTCTGG -3'
X1B 5'-CCAGAAGAACAGCCGGCTAGCGCGCGTCGACGAGC -3'

SEQ ID NOS: 36 and 37 are primers used to amplify a mutated ORFX2 fragment.
X2A 5'-GGAACGGGAGGCAGAGCAGGCTAGCTCATCGGCGGGCCCTTCG -3'
X2B 5'-GGGCCCGCCGATGAGCTAGCCTGCTCTGCCTCCCGTTCC -3'

SEQ ID NOS: 38 and 39 primers used to amplify a mutated ORFY fragment.
YA 5'-GTGTTGATCCAGCTAGCGGGCGCGATGCGGTGAAG -3'
YB 5'-TTCACCGCATCGCGCCCGCTAGCTGGATCAACACC -3'

SEQ ID NOS: 40 and 41 are primers used to make a probe to identify *A. luteus* homologs.

ORFYF: 5'- AGAGGAGCCGAGCGATGAG -3'
ORFYR: 5'- CGTACCAGATCAGCAGCATC -3'

SEQ ID NOS: 42 and 45 are primers used for *M. luteus* genomic walking.

GSP1F: 5'-TTCATGGACGTGCCCAGCAGCGTTGCCA-3'
GSP2F: 5'-AGGTGGGCGAAGTCCGTGTAGAGGAAG-3'
GSP1F2: 5'-AAGTAGGTGCGTCCGAGCTGGTCGTGGT-3'
GSP2F2: 5'-GTCCGCGCCGAGATCCCGCAGGAAGTT-3'